

PHOSPHONATE METABOLISM IN TETRAHYMENA

A THESIS

submitted for the degree

of

DOCTOR OF PHILOSOPHY

in the

AUSTRALIAN NATIONAL UNIVERSITY

by

CHI-RONG LIANG

September 1967.



### Errata

<u>Page</u>	<u>Line</u>	<u>Remarks</u>
1	10	"HOriguchi" should be "Horiguchi".
2	7	"phosphorus acid" should be "phosphorous acid".
24	4	"Los-phase" should be "Log-phase".
29	7	"present" should be "presence".
30	3	"phosphonolipids" should be "phospholipids".
38	11	"resports" should be "reports".
43	4	"cepahlin" should be "cephalin".
44	27	"primary" should be "primarily".
49	28	"absorbable" should be "adsorbable".
64	12	"amino acid" should be "amino acids".
87	20	".... AEP. In which ..." should be ".... AEP in which ...".

Table II.3, line 5, "containined" should be "containing".

Table V.5            There is need to insert " $\mu$ " in the 3rd heading so that  
"moles" becomes " $\mu$ moles".



STATEMENT

This thesis embodies the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, the Australian National University, from March 1964 to September 1967, during the tenure of an Australian National University Research Scholarship.

Candidate's signature:

Chun-ying Liang

## ACKNOWLEDGEMENTS

I wish to express my special thanks to my supervisor, Dr. H. Rosenberg, for his advice and discussions throughout this work.

### STATEMENT

The regulations of the Australian National University require that a statement be made describing which parts of the work in this thesis have been carried out by myself.

Some of the isotopic compounds and enzyme preparations described in this thesis were provided by members of this Department, as acknowledged in the text.

The experiments described in Chapter IV under D (iv) were carried out in partial collaboration with Dr. H. Rosenberg.

The remainder of the work was carried out by myself, under the supervision of Dr. Rosenberg.

I wish to thank Dr. R. Smith for his identification of the eluents used in part of this work and to Drs. D.J. Brown and J.M. Swan for their valuable discussions concerning some topics of phosphonate chemistry dealt with in this work.

Finally, I should like to thank Mrs. P. Smith for her help in the preparation of the thesis, Mrs. P. Smith for her care and skill in typing, Mr. R. Wilson and staff and Mrs. A. Aldrich for the preparation of the photographic prints.

Candidate's signature:

Chi-rong Liang

## ACKNOWLEDGEMENTS

I wish to express my special thanks to my supervisor, Dr. H. Rosenberg, for his helpful advice and discussions throughout this work.

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My thanks are also due to my many departmental colleagues who have generously given of their time and specialised knowledge. In particular I should like to thank Dr. D.C. Shaw for his assistance in carrying out amino acid analysis and Drs. J.F. Morrison and D.I. Magrath for their helpful discussions.

Acknowledgements are also due to Dr. B. Smith for his identification of the slugs used in part of this work and to Drs. D.J. Brown and J.M. Swan for their valuable discussions concerning some topics of phosphonate chemistry dealt with in this work.

Finally, I should like to mention those who have helped me in the preparation of this thesis; Miss P. Smith for her care and skill in typing, Mr. R. Westen and his staff and Mrs. A. Aldrich for the preparation of the photographic prints.

## PREFACE

The discovery of 2-aminoethylphosphonic acid in protozoa several years ago constituted the first demonstration of carbon-bound phosphorus in nature. Since then the biosynthesis of the carbon-phosphorus bond presented a continuing challenge to biochemists.

This work was undertaken in the hope of elucidating the mechanism of this completely unprecedented type of biosynthesis.

The investigations can be grouped into three major headings:

1. A search for the site and mechanism of C-P bond formation in Tetrahymena.
2. In vitro studies of the biosynthesis of the phosphonic analogue of phosphatidylethanolamine, i.e. diacylglycerylaminoethylphosphonic acid.
3. A short comparative study of C-P bond formation in the slug.

The structure of each chapter in this thesis follows, as far as possible, the Policy and Instructions of the Biochemical Journal. Each Figure and Table is presented on a separate page, and appear immediately following the page on which first reference to it has been made.

In addition to standard abbreviations for units of mass, volume and time, the following abbreviations will be used:

AEP	2-aminoethylphosphonic acid
APP	2-amino-3-phosphonopropionic acid
PE	phosphorylethanolamine
DNP	2,4-dinitrophenol
BHT	4-methyl-2,6-di- <u>tert</u> -butylphenol
CMP	cytidine-5'-monophosphate
NEM	N-ethylmorpholine

CDP	cytidine-5'-diphosphate
CTP	cytidine-5'-triphosphate
CDPE	cytidine diphosphate ethanolamine
CDP-choline	cytidine diphosphate choline
CMP-AEP	cytidine monophosphate 2-aminoethyl-phosphonate
ATP	adenosine-5'-triphosphate
EDTA	ethylenediaminetetraacetate
DEAE-cellulose	diethylaminoethyl-cellulose

For the sake of simplicity, enzymes have been referred to in the text of the Thesis by their trivial names. A list of the enzymes that have been mentioned follows, together with the numbers by which they are designated in the Recommendations (1964) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes.

<u>NUMBER</u>	<u>SYSTEMATIC NAME</u>	<u>TRIVIAL NAME</u>
1.3.99.1	Succinate:FAD oxidoreductase	Succinate dehydrogenase
2.7.7.14	CTP:phosphorylethanolamine cytidyltransferase	PE-cytidyltransferase
2.7.7.15	CTP:phosphorylcholine cytidyltransferase	phosphorylcholine cytidyltransferase
	CTP:AEP cytidyltransferase	AEP-cytidyltransferase
2.7.8.1	CDPE:1,2-diglyceride PE transferase	PE-glyceride transferase
	CMP-AEP:1,2-diglyceride AEP transferase	AEP-glyceride transferase
3.1.1.7	Acetylcholine hydrolase	Acetylcholine- esterase
3.1.1.8	Acylcholine acyl-hydrolase	Cholinesterase



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<u>GENERAL INTRODUCTION</u>	



## CHAPTER I. GENERAL INTRODUCTION

### 1. The Chemistry and Some Biological Effects of the Synthetic Phosphonates.

#### A. Chemistry of the synthetic phosphonates

Phosphonates are the compounds which contain a covalent bond between the carbon and phosphorus atoms. They differ from phosphate esters (C-O-P bond) by their high resistance to acid or alkaline hydrolysis. For instance

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(Finkelstein, 1946; Kozolapoff, 1947; Chavane and Hackspill, 1947; Chavane, 1943). Recently, Chambers and Isbell (1964) published the syntheses of some amino-alkyl-phosphonates, such as L-aminoethylphosphonate, L-amino-2-phenyl-ethylphosphonate, aminomethylphosphonate, 2-amino-4-phosphono-butyrate and 2-amino-3-phosphono-propionate.

In the search for an approach to the formation of the C-P bond *in vivo*, it may be profitable to examine the known methods for making phosphonates chemically (cf. Kozolapoff, 1950). The methods are divided into three categories: (I) those involving reactions that are possible in a biological system; (II) those widely employed by biochemists; and (III) those of interest, but of little importance in biochemistry.

## CHAPTER I. GENERAL INTRODUCTION

## 1. The Chemistry and Some Biological Effects of the Synthetic Phosphonates.

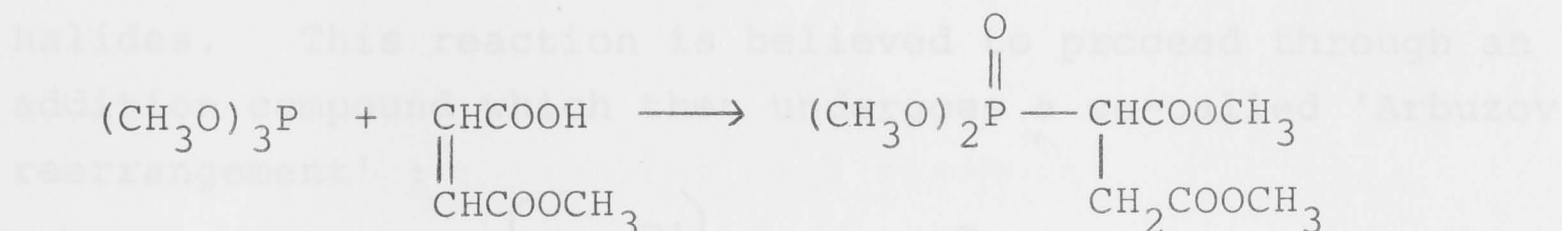
## A. Chemistry of the synthetic phosphonates

Phosphonates are the compounds which contain a covalent bond between the carbon and phosphorus atoms. They differ from phosphate esters (P-O-C bond) by their high resistance to acid or alkaline hydrolysis. For instance, treatment of 2-aminoethylphosphonic acid (AEP) with 5N sodium hydroxide at 120° for 8 hours (Horiguchi and Kandatsu, 1959) or hydrolysis with 6N hydrochloric acid at 120° for 72 hours does not split the phosphonic (C-P) bond (Horiguchi and Kandatsu, 1962). Aromatic aminophosphonates are reported to be even more stable than the alkyl aminophosphonates (Freedman and Doak, 1957).

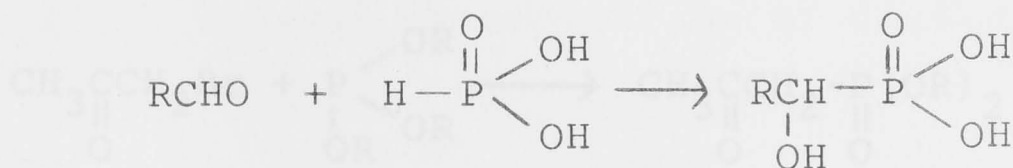
Of the many synthetic phosphonates, 2-aminoethylphosphonate was synthesised during the nineteen-forties (Finkelstein, 1946; Kosolapoff, 1947; Chavane and Hackspill, 1947; Chavane, 1949). Recently, Chambers and Isbell (1964) published the syntheses of some amino-alkyl-phosphonates, such as 1-aminoethylphosphonate, 1-amino-2-phenyl-ethylphosphonate, aminomethylphosphonate, 2-amino-4-phosphono-butyrate and 2-amino-3-phosphono-propionate.

In the search for an approach to the formation of the C-P bond in vivo, it may be profitable to examine the known methods for making phosphonates chemically (cf. Kosolapoff, 1950). The methods are divided into three categories : (I) those involving reactions that are possible in a biological system; (II) those widely employed by biochemists; and (III) those of interest, but of little importance in biochemistry.

I. The route of phosphonate synthesis perhaps most likely in a biological system involves addition of a phosphite ester to an  $\alpha,\beta$ -unsaturated compound. Thus trimethyl phosphite and monomethyl maleate yield at least 64% (judged by gas chromatography) of the tetramethyl phosphonosuccinate (Hindersinn and Ludington, 1965).

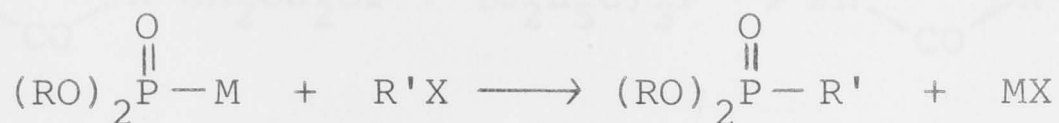


Another such reaction involves addition of phosphorus acid or a phosphite to an aldehyde or ketone as shown below (Marie, 1903a; 1903b; 1904).

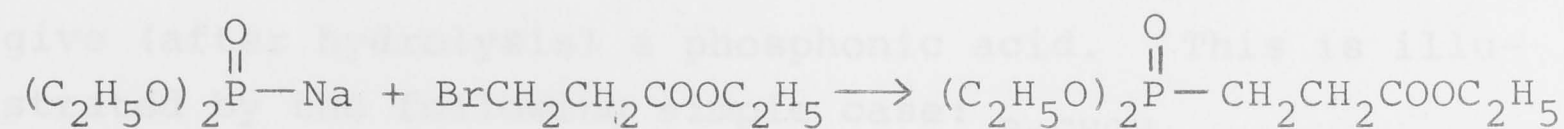


II. Syntheses which are widely used by biochemists:

i. Reaction of sodium or potassium salt of diethyl or dibutyl phosphite with an alkyl or aryl halide:



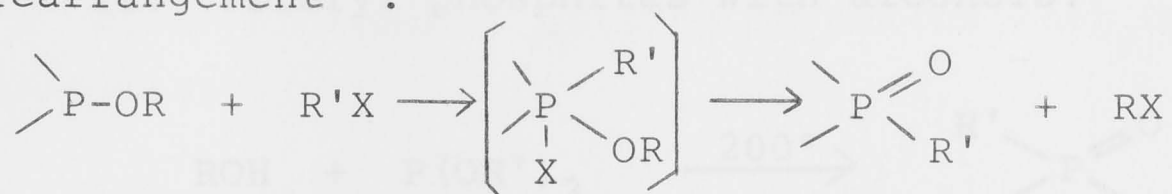
In this way, Nylen (1926) and Finkelstein (1946) made triethyl 3-phosphonopropionate and its  $\underline{\text{C}}$ -amide; Hofmann degradation of the latter furnished 2-aminoethylphosphonate (AEP):





AEP

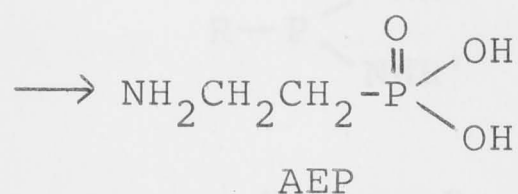
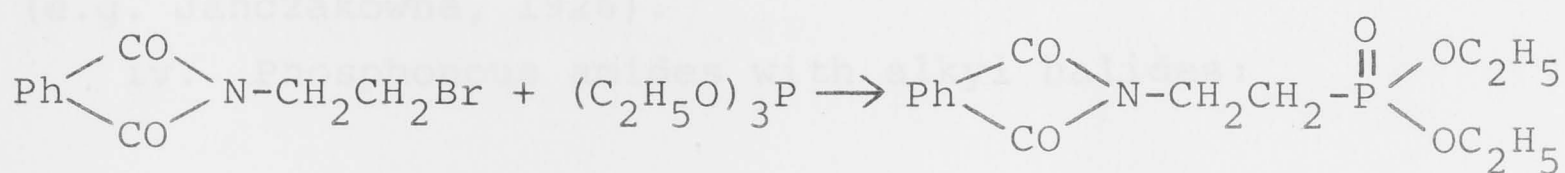
ii. The reaction of tertiary phosphite with organic halides. This reaction is believed to proceed through an addition compound which then undergoes a so-called 'Arbuzov rearrangement' :



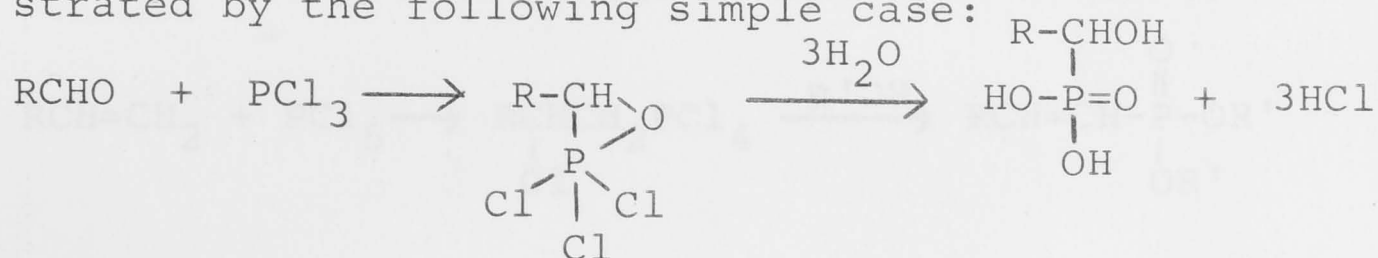
In this way, 1-phosphonoacetone was prepared (Machleidt and Strehlke, 1964) as shown below:



2-aminoethylphosphonate was prepared in a similar way (Kosolapoff, 1947):

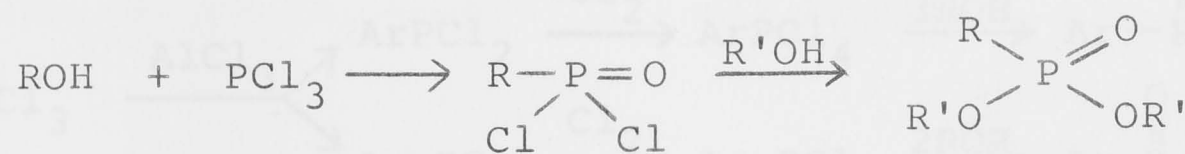


iii. The reaction between aldehyde or ketone and Phosphorus trichloride (Conant and Macdonald, 1920) to give (after hydrolysis) a phosphonic acid. This is illustrated by the following simple case:



III. Other routes to phosphonates which are of less interest in biochemistry but are briefly summarized below:

i. Phosphorus trichloride with alcohols:



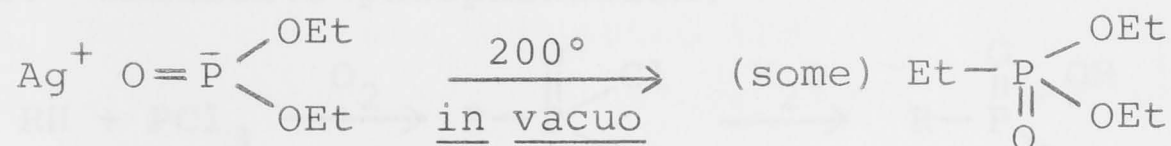
(e.g. Hatt, 1933).

ii. Triaryl phosphites with alcohols:



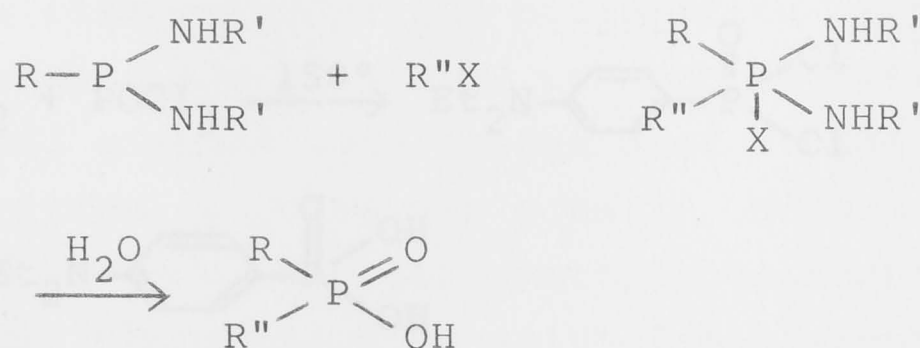
(e.g. Milobendzki and Szulgin, 1917).

iii. Thermal decomposition of dialkyl phosphite salts:



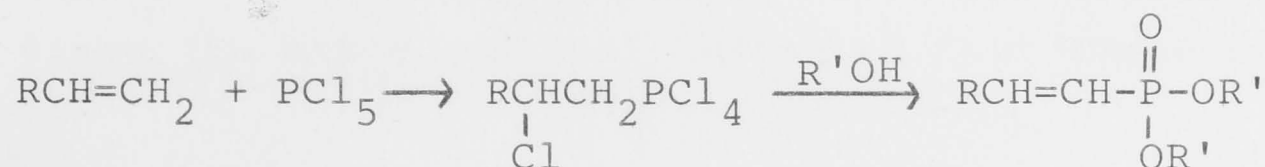
(e.g. Janczakowna, 1926).

iv. Phosphonous amides with alkyl halides:



(e.g. Pope and Gibson, 1912).

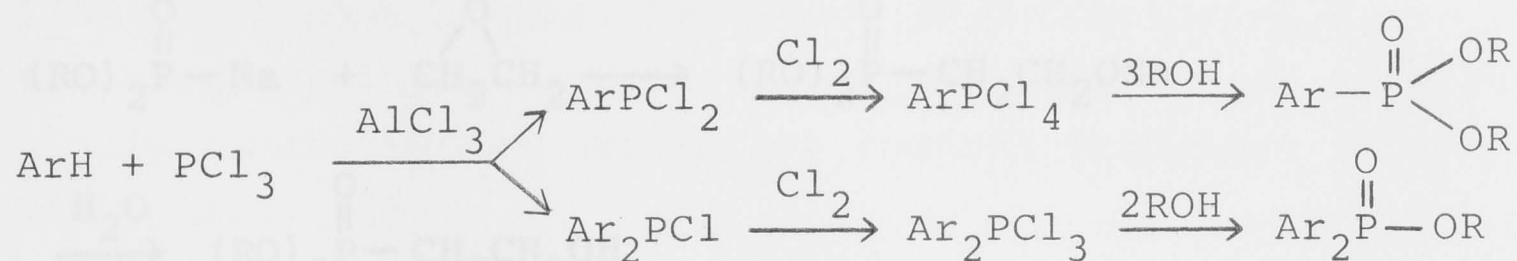
v. Phosphorus pentachloride with olefines:





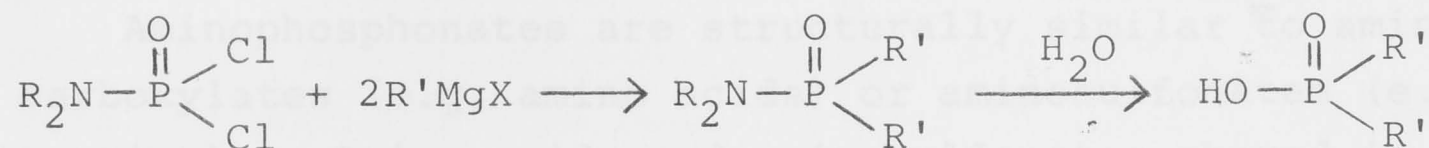
(e.g. Bergman and Bondi, 1930, 1933; Kosolapoff, 1946).

vi. Phosphorus trichloride with aromatic compounds in presence of aluminium chloride:



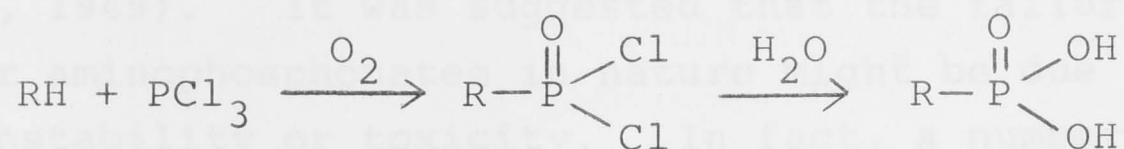
Good yields result (e.g. Dye, 1948).

vii. Phosphorus halides or esters with Grignard reagents:



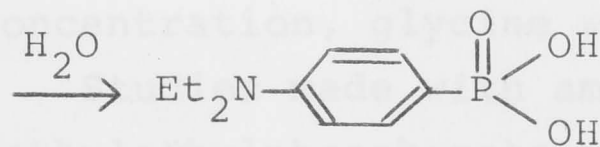
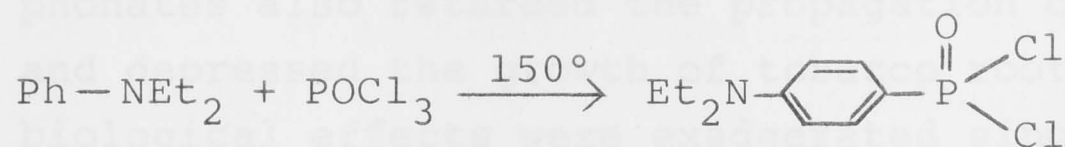
(e.g. Kosolapoff, 1942).

viii. Oxidative phosphonation:



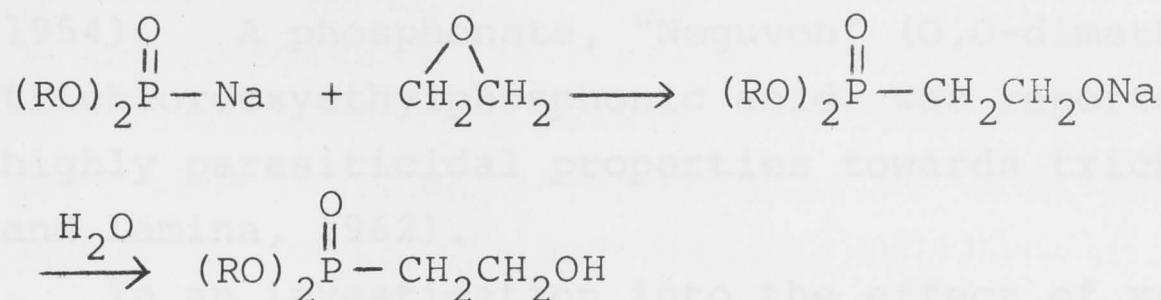
(e.g. Clayton and Jensen, 1948).

ix. Phosphorus halides with NN-dialkylanilines:



(e.g. Bourneuf, 1923).

x. Reaction of a dialkyl phosphite with an olefine oxide:



(Chelintsev and Kuskov, 1946).

#### B. Some biological effects of phosphonates

Aminophosphonates are structurally similar to amino-carboxylates (e.g. amino acids) or aminosulfonates (e.g. taurine). Amino acids and aminosulfonates abound in nature, and the possible existence of aminophosphonates in nature had been predicted (Chavane and Hackspill, 1947; Chavane, 1949). It was suggested that the failure to discover aminophosphonates in nature might be due to their instability or toxicity. In fact, a number of aminophosphonates have been found to be toxic, in high concentration, to silk worms and chick embryos (Ryzhkov et al., 1954). In very high concentrations these phosphonates also retarded the propagation of tobacco virus and depressed the growth of tobacco rootlets. But those biological effects were exaggerated since, at the same concentration, glycine was equally inhibitory.

Studies made with aminomethylphosphonate, 1-amino-1-methylethylphosphonate, 1-amino-2-methyl-propylphosphonate, 1-amino-3-methyl-butylphosphonate, 1-amino-4-methyl-amylphosphonate and 1-amino-1-phenyl-methylphosphonate on tobacco roots, tobacco virus, silk worm and influenza virus (in chick embryos) indicated that these compounds



could not be metabolized by the organisms. Liver, kidney and plant tissues also failed to transform the carbon-linked phosphorus into inorganic phosphate (Ryzhkov et al., 1954). A phosphonate, "Neguvon" (O,O-dimethyl-2,2,2-trichlorooxyethylphosphonic acid) was reported to possess highly parasitocidal properties towards trichinae (Schoop and Lamina, 1962).

In an investigation into the effect of various substances on the uptake of methionine by Escherichia coli, it was found that aminomethylphosphonate was a weak inhibitor of methionine uptake, although aminomethylsulfonic and l-aminoethylsulfonic acids stimulated the methionine uptake (Melchoir, Klioze and Klotz, 1951). Some phosphonates have been reported to inhibit certain enzymes. In testing a series of halogenated aromatic phosphonates and phosphinates ( $R-PO_2H$ ) as well as their esters against cholinesterase, it was found that many of these compounds possessed anti-cholinesterase activity (Freedman, Tauber, Doak and Magnuson, 1953). In addition to human plasma cholinesterase, inhibition was also obtained with pig brain acetylcholinesterase and insect brain acetylcholinesterase (Tauber and Petit, 1953). 3-Phosphonopropionate was reported to be a competitive inhibitor of succinate dehydrogenase in the ciliate Tetrahymena (Seaman, 1952). Incidentally, this phosphonate did not inhibit the succinate dehydrogenase of rat heart, liver, brain or skeletal muscle.

A large number of aromatic phosphonates have been tested for antibacterial activity (Thayer, Magnuson and Gravatt, 1953). Of twenty-nine aromatic phosphonates tested, fifteen compounds showed significant antibacterial activity.

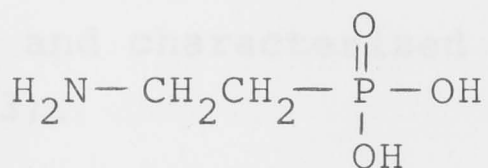
Antiviral activity of phosphonates has also been

reported. The effect of p-nitrobenzylphosphonate on the propagation of encephalomyocarditis virus (EMC) was investigated both in vitro and in vivo, and the compound was found to possess antiviral activity (Inglot, Kochman and Mastalerz, 1965). Marked antiviral activity was also exhibited by other phosphonic acids with benzyl groups (Kochman, Mastalerz and Inglot, 1965).

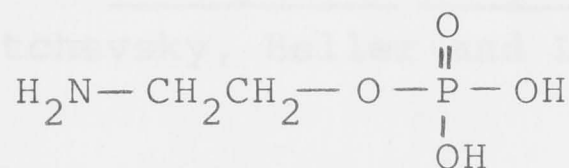
In contrast to these reports of inhibitory effects of phosphonic acids, 1-naphthylmethylphosphonic acid has been reported to be a growth regulator to plants (Freedman and Doak, 1957).

## 2. Naturally Occurring Phosphonates

The existence of phosphonates in nature was not discovered until 1959 when Horiguchi and Kandatsu (1959, 1960) isolated 2-aminoethylphosphonic acid (AEP) from the acid hydrolysate of the ether-ethanol soluble fraction of sheep rumen protozoa. This compound (formula I) is the phosphonate-analogue of phosphorylethanolamine (PE) (formula II).



I



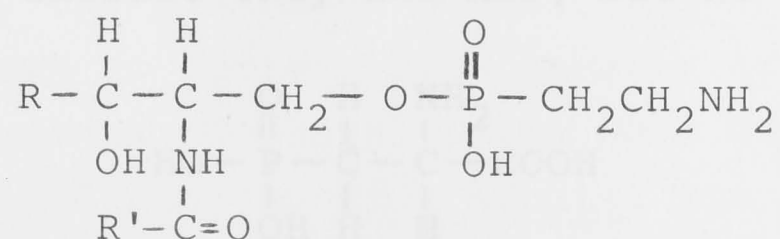
II

The phosphorus of compound I was found to be more stable to hydrolysis than that of phytin; it could not be released by hydrolysis in 6N mineral acids at 120° for 72 hours. Mineralization of this compound could not be accomplished by the methods of Fiske and Subbarow (1925) or Allen (1940). Only complete oxidation by heating in  $\text{H}_2\text{SO}_4\text{-HClO}_4$  or  $\text{H}_2\text{SO}_4\text{-HNO}_3$  mixtures at 360° was effective.

In order to study the distribution of AEP in nature, Kandatsu and Horiguchi (1962) analysed various microorganisms, animal and plant tissues for the possible existence of AEP. While no AEP was found in yeasts, fungi or animal and plant tissues, appreciable amounts were present in some bacteria. The highest concentration was found in the ciliate Tetrahymena, where AEP accounted for 13-15% of the total phosphorus. These authors also grew Tetrahymena in axenic cultures and proved that this organism did synthesize the C-P bond under aerobic conditions.

AEP was also isolated in crystalline form from the hydrolysate of ether-ethanol extracts of the sea anemone Anthopleura elegantissima (Kittredge, Roberts and Simonsen, 1962). The glycerol ester of this phosphonate was also claimed to be present in the mild alkaline hydrolysate of the same material. However, the isolated compound was quite unstable and is likely to be an AEP derivative other than the glycerol ester (Horiguchi, 1966).

A new sphingolipid, ceramide aminoethylphosphonate (formula III) was also isolated from Anthopleura elegantissima and characterized (Rouser, Kritchevsky, Heller and Lieber, 1963).



III

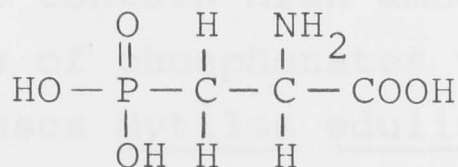
R : alkyl group of long chain alcohol

R' : alkyl group of fatty acid

This appeared to be the first reported instance of the natural occurrence of a sphingolipid with a free amino group and the first isolation of a pure phospholipid with a carbon-phosphorus bond. As no sphingomyelin was found in this organism, this phosphosphingolipid completely replaced the sphingomyelin which is widely distributed in the tissues of other animals. The content of ceramide aminoethylphosphonic acid in that organism was reported to be 9.3% of the total lipid.

AEP has also been isolated from a hydrolysate of insoluble proteinaceous material as well as in an aqueous ethanol and chloroform extract of the sea anemone Metridium dianthus (Quin, 1964). This compound amounted to 1.1% of the insoluble protein fraction and 0.99% of total dry materials. It was suggested that this compound was incorporated into the protein structure of the sea anemone. De Koning (1966) reported that AEP was present in the abalone (Haliotis midae).

Another phosphonic acid, 2-amino-3-phosphonopropionic acid (APP) (formula IV) was isolated from the aqueous ethanol extract of the zoanthid Zoanthus sociatus and the ciliate Tetrahymena pyriformis (Kittredge and Hughes, 1964). In the case of Zoanthus, APP was found in the aqueous ethanol extract only and AEP, but no APP was found in the



IV

lipid fraction. In Tetrahymena, APP was present in both the aqueous-ethanol extract and the residue, but was absent



from the lipid fraction. The amount of APP in Tetrahymena was reported to be 450  $\mu$ moles per g of wet cells in the aqueous-ethanol extract while 125  $\mu$ moles was present in the residue. Since the rapid addition of chilled ethanol to the cell pellet in the centrifuge tubes increased the Yield of APP, it may well be a short-lived intermediate in Tetrahymena.

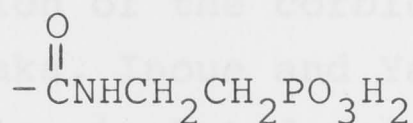
AEP in Tetrahymena was reported to be present in both the free state and in a bound form; the latter was largely in the lipid fraction but considerable amounts were also present in the lipid-extracted residual fraction (Rosenberg, 1964). The residue-bound AEP was stable to proteolytic enzymes. It was suggested that AEP was associated with macromolecular complexes and formed structural material of the cells.

A more detailed investigation of the distribution of phosphonic acids in marine animals, as well as of the nature of the residue-bound AEP in these animals, was reported by Quin (1965). The sea anemone Tealia felina was found to be the richest source of phosphonates which amounted to 410 mg P per 100 g of dry tissues. As much as 50% of the phosphorus in this organism was reported to be carbon-bound. In addition to Tealia felina and Metridium dianthus the gastropod mollusc Busycon canaliculatum was also shown to contain high amounts of phosphonate-P. Small amounts of phosphonates were also found in the bivalve molluscs Mytilus edulis, the quahog Venus mercenaria and the starfish Asteria forbes. A study of residue-bound AEP in Metridium dianthus indicated that, when the residue was solubilized in 1 N NaOH, the trichloroacetic acid precipitate from the solution contained phosphonates. After pepsin digestion, 90% of total residue-bound AEP

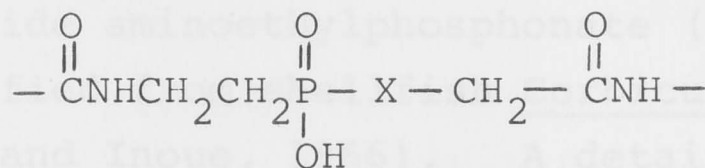
was located in polypeptides while only 10% was released in short chain peptides. The minimum molecular weight of the polypeptide was found to be 2840. Dinitrophenylation, followed by acid hydrolysis of the DNP-polypeptide, did not yield DNP-AEP, indicating that none of the AEP residues in the peptide mixture possessed a free amino group.

It was suggested that AEP might be bound in the polypeptide in the following ways:

- (1) through the amino group:



- (2) through both amino and phosphonic groups:



(X = NH or O)

However, the reported results did not provide a complete solution to this problem.

The occurrence of phosphonates in mammalian tissues was first reported by Shimizu, Kakimoto, Nakajima, Kanazawa and Sano (1965), who isolated free AEP from the acid-soluble fraction of bovine brain tissue. The content of free AEP in bovine brain was reported to be 2  $\mu\text{g}$  per g wet tissue, while that of lipid-bound AEP was less than 1  $\mu\text{g}$  per gram wet tissue. As there has been no demonstration of actual biosynthesis of phosphonates in mammalian tissues, it is most probable that AEP isolated from bovine brain originated from rumen protozoa. It has

been shown (Kandatsu and Horiguchi, 1965) that AEP-containing protozoa in the sheep reticulorumen could be digested and absorbed in the abomasum and the intestine. Free AEP was isolated from goat liver in yields amounting to 58.4 mg per total liver (742 g), while lipid-bound AEP amounted to only 8.8 mg. It was suggested that AEP originating from rumen protozoa may be distributed in various tissues of the host, as well as in secretions and excreta. It is therefore possible that humans ingest some AEP through dairy products. Ceramide aminoethylphosphonate had been isolated from the pyridine-insoluble lipid fraction of the corbicular, Corbicular sandai (Hori, Itasaka, Inoue and Yamada, 1964). This phosphonolipid was also isolated from five other species of shellfish. It accounted for 53-78% (depending on the species) of the total pyridine-insoluble lipids. A large quantity of ceramide aminoethylphosphonate (2.5 g) was isolated and purified from shellfish Corbicular sandai (Hori, Itasaka and Inoue, 1966). A detailed analysis of this lipid was given for the first time.

The distribution of phosphonates in nature is summarized in Tables I.1, I.2 and I.3.

The phosphonic analogue of phosphorylcholine, 2-trimethylaminoethylphosphonic acid as well as 2-methylaminoethylphosphonic acid have been isolated from the sea anemone Anthopleura xanthogrammica and tentatively identified (Kittredge and Isbell, 1967). The discovery of phosphonic analogues of phosphorylethanolamine, phosphorylcholine as well as 2-methylaminoethylphosphate indicated the possible existence of the phosphonic analogues of phosphatides in nature. So far, the only phosphonic analogue of a phospholipid isolated from natural sources appears to be a ceramide aminoethylphosphonate (Rouser



TABLE I.1. Relative content of phosphonate in marine animals (in mg phosphonate-P per 100 g of dry tissue)

<u>Tealia felina</u>	410
<u>Metridium dianthus</u>	302
<u>Busycon canaliculatum</u>	215
<u>Mytilus edulis</u>	76
<u>Asterias forbesi</u>	32
<u>Venus mercenaria</u>	28

[From L.D. Quin, Biochemistry, 4, 324 (1965)]

TABLE I.2. Ceramide aminoethylphosphonate content of shellfish lipids

Species	% Ceramide-AEP in total pyridine-insoluble lipids
<u>Corbicula sandai</u>	65
<u>Corbicula japonica</u>	66
<u>Unio biwae</u>	78
<u>Inversidens hirasei</u>	67
<u>Anodonta lauta rostrata</u>	53
<u>Cristaria plicata</u>	65

[From T. Hori et al., J.Biochem.(Japan) 56, 477 (1964)]

TABLE I.3. AEP content of various species

Name	AEP content (% AEP-P in total P)
Slug	22.9
<u>Tetrahymena pyriformis</u> W	10.6 - 15.0
Rumen ciliates	3.4 - 6.5
Animal plankton	3.2
Rumen bacteria	1.0
<u>Paramecium</u> K <sub>3</sub> 2	0.82
<u>Selenomonas</u>	0.46-0.65
<u>Coryn. facians</u>	0.25
<u>Panagrellus redivivus</u>	0.23
<u>Bacillus subtilis</u>	0.15-0.17
<u>Agrob. tumefaciens</u>	0.14
<u>Euglena gracilis</u>	0.10
<u>E. coli</u>	0.06-0.07
<u>Cl. butyricum</u> (phosphate media)	0.04
<u>Cl. butyricum</u> (hypophosphate media)	0.29-0.40

[From M. Horiguchi, J.Agr.Chem.Soc.Japan, 46, R25 (1966)]

et al., 1963; Hori et al., 1964).

The reports of widespread occurrence of phosphonates in natural lipids has stimulated the chemical synthesis of phosphonic analogues of the well known phospholipids. Phosphonic analogues of phosphatidylethanolamine with aliphatic long chain residues in ester, as well as ether, linkage have been prepared by Rosenthal and Pausada (1964, 1965) and by Baer and Stanacev (1964, 1965a).

The syntheses of a phosphonic acid analogue of lecithin (Baer and Stanacev, 1965b) and the diether phosphonate analogue of phosphatidic acid (Rosenthal et al., 1964) have also been reported.

This field was recently reviewed by Baer (1966).

A direct method for the determination of phosphonates has not yet been devised. AEP determination is usually based on the stability of its C-P bond to hydrolysis. Since all ester phosphates are hydrolysed (6N HCl, 120°, 48 hours) to orthophosphate, the assay simply involves the separation of the phosphonate from orthophosphate following hydrolysis. When the phosphonate is AEP, the matter is simplified because of the considerable charge difference between the two compounds. Dowex-1 or Dowex-50 ion exchange resins are widely used for the separation of those two compounds (Horiguchi and Kandatsu, 1959; Kittredge et al., 1962).

### 3. The Metabolism and Biosynthesis of Phosphonates

#### A. The metabolism of phosphonates

The carbon-phosphorus bond of the phosphonate is much more stable, both chemically or enzymically, than the phosphate ester bond.

In vivo studies with certain microorganisms or higher animals which could utilize phosphonates to some extent have been reported. Escherichia coli (Crookes strain)

was shown to grow in media which contained methyl- or ethylphosphonic acid as the sole source of phosphorus (Zeleznick, Myers and Titchener, 1963). The growth of ten different microorganisms on eight different amino-alkylphosphonates was also reported (Harkness, 1966). The result showed that, of 10 organisms tested, 9 were able to grow on at least one of the eight phosphonates. The growth was most rapid on AEP and was comparable to the growth on orthophosphate. The ability to catabolize the carbon-phosphorus bond was shown to be widespread among bacteria. Therefore it is clear that an enzyme (or enzymes) capable of breaking down the C-P bond, exist in various organisms. Escherichia coli 307, Escherichia freundii and Mycobacterium phlei have also been reported to grow on synthetic media in which inorganic phosphate was replaced by ethylphosphonate or 1-phosphono-2,3-propane-diol (Mastalerz, Wieczorek and Kochman, 1965). The growth of M. phlei in that medium was slower, and the phosphorus content of the cells was lower, than in phosphate-supplemented medium. Although evidence was presented that M. phlei could split the C-P bond in vivo, attempts to demonstrate the C-P bond cleavage in cell-free homogenates of M. phlei were unsuccessful.

Rosenberg and La Nauze (1967) showed that several strains of Bacillus cereus were capable of utilizing AEP as a sole source of phosphorus. They also showed the breakdown of AEP with the production of orthophosphate by cell-free extracts of Bacillus cereus (La Nauze and Rosenberg, personal communication).

Reports of AEP metabolism in higher animals are restricted to its transportation and accumulation. No evidence of the breakdown of phosphonate to phosphate in higher animals is available at present. It was reported



that AEP fed orally to rats was accumulated in the liver (Kandatsu, Horiguchi and Tamari, 1965). It was also shown that four days after radioactive-AEP was given to rats, 60% of the total dose (338  $\mu$ moles) was absorbed through the intestine and 33% of the total dose was actually retained in the body. Analysis showed a high amount of AEP in the liver, where it was lipid- and residue-(protein-aceous) bound. It was also reported that when a sheep was injected with radioactive AEP, the compound accumulated predominantly in the liver, both in the lipid-bound and acid-soluble forms (Horiguchi, 1966).

Protozoa of the ruminal microflora are ingested by the host in the course of the normal digestive cycle and it can be reasonably expected that some amounts of AEP will find their way into the tissues of ruminants. The presence of AEP in the tissues of the sheep, but not of the rat, has been demonstrated convincingly by the use of the isotope dilution technique with [ $^{32}$ P]AEP (Kandatsu et al., 1965; Kandatsu and Horiguchi, 1965).

The incorporation of AEP into the lipid of the housefly, Musca domestica has also been reported (Bridges and Ricketts, 1966). When houseflies were fed AEP, an AEP-containing phospholipid was isolated and identified by thin layer chromatography as dialkylglyceryl-(2-aminoethyl)phosphonic acid. The incorporation of AEP into phospholipids of the housefly, rat or sheep was attributed to the low specificity of the enzymes responsible for phospholipid synthesis.

A similar course of events could occur if humans were fed AEP. In fact, the existence of AEP in some edible bivalves (Quin, 1965) and in the abalone (De Koning, 1966) is now established and the possible occurrence of AEP (of ruminal origin) in dairy products has been suggested

(Horiguchi, 1966). Thus man may ingest appreciable amounts of AEP which would be absorbed through the intestine and accumulated in the liver in a manner similar to that observed under experimental conditions in the rat and sheep.

#### B. The biosynthesis of phosphonates

Little headway has been made so far on the biosynthetic pathways by which the phosphonic bond (C-P) is formed. The chemical methods for the formation of the C-P bond have been summarised above. The important role of phosphites in the chemical synthesis of phosphonates is well established (see above). However, the involvement of phosphite in phosphonate biosynthesis has not been demonstrated. It has been claimed that the presence of phosphite increased the incorporation of ethanolamine into AEP in a cell free homogenate of Tetrahymena (Horiguchi, 1966), but the claim was not accompanied by experimental evidence.

It was reported that AEP synthesis occurred in Tetrahymena during the logarithmic growth and ceased in stationary cells (Rosenberg, 1964). Lipid-AEP was shown to behave as an end product of metabolism and it was suggested that it may be associated with the structural elements of the cells. The important finding was that, in the cells incubated in the presence of [ $^{32}$ P]orthophosphate, the specific radioactivity of [ $^{32}$ P]AEP was highest in the lipid-bound fraction and this suggested that the site of C-P bond formation in Tetrahymena may be in a phospholipid molecule. The finding of 2-amino-3-phosphonopropionic acid in the Zoanthus and Tetrahymena (Kittredge and Hughes, 1964) suggested that this compound may be the precursor of 2-aminoethylphosphonic acid, since the

biosynthetic route of many amines leads through the decarboxylation of the corresponding amino acids. However, the specific activities of these compounds after [ $^{32}\text{P}$ ]orthophosphate incorporation were below that of AEP which makes it unlikely that 2-amino-3-phosphonopropionic acid is the precursor of AEP.

Segal (1965) postulated a scheme for the formation of the C-P bond starting from phosphatidylethanolamine through a phosphoramidic acid rearrangement. Again, no experimental evidence was presented.

The present work is concerned with the metabolism of phosphonates in Tetrahymena. This includes phosphonate bond biosynthesis and the metabolism of AEP as well as its derivatives by the cell. Although the problem of C-P bond biosynthesis is still unsolved, much information was obtained and a mechanism has been postulated. In connection with the metabolism of phosphonates in Tetrahymena, the presence of a phosphonic analogue of cepahlin has been demonstrated. The synthesis both in vitro and in vivo of a phosphonate-containing phosphatide from AEP through a cytidine nucleotide-bound intermediate has also been shown.



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CHAPTER II. THE BIOSYNTHESIS OF 2-ANTHOETHYLPHOSPHONATE-  
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CHAPTER II. THE BIOSYNTHESIS OF 2-AMINOETHYLPHOSPHONATE-  
CONTAINING LIPIDS IN TETRAHYMENA

Summary

1. The observation that AEP of the lipid and of the residual fraction of Tetrahymena was formed more rapidly than that of the free AEP has been confirmed.
2. Further investigations of the biosynthesis of AEP in Tetrahymena showed that the site of first synthesis lies within a lipid molecule which is saponifiable (glyceride).
3. The presence of glyceryl-aminoethylphosphonic acid has been demonstrated in the products of alkaline hydrolysis of Tetrahymena lipid.
4. Phosphatidylethanolamine and its phosphonic analogue, "AEP-cephalin", in the molecular ratio of approximately 13 to 1, have been isolated from Tetrahymena lipids.

## 1. Introduction

Work published on naturally occurring phosphonates has mostly been confined to the distribution of these compounds. While some reports have dealt with the metabolism of phosphonates (Zelevnick et al., 1963; Rosenberg, 1964; Kandatsu et al., 1965; Mastalerz et al., 1965; Rosenberg and La Nauze, 1967), the actual formation of the C-P bond has received little attention.

Rosenberg (1964) reported that, when [ $^{32}\text{P}$ ]orthophosphate was added to a growing culture of Tetrahymena, the specific radioactivity of [ $^{32}\text{P}$ ]AEP in the cells increased most rapidly in the lipid-bound AEP and least in the free AEP. This indicated that the rate of AEP formation was highest in the lipid fraction and suggested a special role for Tetrahymena lipid in phosphonate bond formation. Although the formation of AEP-containing lipids from free AEP in vivo has been reported (Kandatsu et al., 1965; Bridges and Ricketts, 1966), this was considered to be the result of the low specificity of enzymes with respect to substrate in phospholipid biosynthesis.

It is the purpose of this chapter to demonstrate a preliminary approach towards the problem of phosphonate bond formation in Tetrahymena. Tetrahymena pyriformis was chosen as the subject of study since it has been shown to synthesize AEP in axenic cultures (Kandatsu and Horiguchi, 1962). Furthermore, the organism could be easily grown in the laboratory under suitable conditions.

Parts of the labelling experiments to be described are similar to those of Rosenberg (1964) except that the cells were harvested from the culture medium and suspended in freshly prepared medium containing [ $^{32}\text{P}$ ]orthophosphate. The incubation time was also shortened. The results of Rosenberg on the rate of AEP formation in the lipid-bound

fraction have been confirmed. Further labelling experiments showed that the saponifiable lipid fraction obtained the highest incorporation of orthophosphate into bound-AEP; glyceryl-AEP was recovered from the saponified lipid of Tetrahymena. Fractionation of Tetrahymena lipid led to the isolation of a phosphonic analogue of phosphatidylethanolamine ("AEP-cephalin") from that fraction. Evidence for the presence of these phosphonates in Tetrahymena will be presented.

## 2. Materials and Methods

### A. Materials

Chemicals used were of analytical grade whenever available. L-Amino acids and certain vitamins used in media were products of Nutritional Biochemicals Co., Cleveland, Ohio. Other media components were purchased from various sources and were of the highest purity available.

Tetrahymena pyriformis Wh-14 (mating type 2, variety 1) was originally obtained from Dr. O.H. Scherbaum and was used throughout.

2-Aminoethylphosphonic acid (AEP) was obtained from Professor A.F. Isbell. AEP labelled with [ $^{32}\text{P}$ ] was prepared from the lipid of Tetrahymena grown in the presence of [ $^{32}\text{P}$ ]orthophosphate for 40 h., as follows: the lipid was extracted and subjected to hydrolysis in 6N HCl as described under "Methods" below. The acid hydrolysate was extracted with ether to remove fatty acids etc., filtered through glass paper and rendered acid-free by repeated evaporation under reduced pressure in a rotatory evaporator. The residue was dissolved in water and the solution applied to a column of Dowex-50 x 4 ( $\text{H}^+$ ). The column was washed with water until the effluent was acid-



free and the AEP was eluted with 2.5N ammonium hydroxide. The effluent was monitored for radioactivity and the radioactive portion was taken to dryness. The residue was re-dissolved in water and applied to a column of Dowex-1 x 2 (acetate). After washing the column with water, the AEP was eluted with 5% (v/v) acetic acid. At this stage, the material was radiochemically pure, in that AEP was the sole radioactive compound, although some minor ninhydrin-reacting components were still present. Pure labelled AEP was prepared from this product by one further passage through Dowex-1 x 2 (formate) using an increasing concentration gradient of formic acid (0 to 0.04 N). The emergent radioactive fraction in this case contained only AEP. From a culture of cells grown in 300 ml of the 'complex medium' (see below) containing 1 mC of [ $^{32}$ P]orthophosphate, the yield of radioactive AEP was about 60  $\mu$ moles with a radioactivity of about 50  $\mu$ C.

[ $^{32}$ P]Orthophosphate was purchased from the Australian Atomic Energy Commission.

Ion exchange resins Dowex-1 x 2 and Dowex-50 x 4 were Fluka A.G. products.

Dipalmitin (mixed isomers 1,2- and 1,3-di-O-palmitylglycerol) and phosphatidylethanolamine were products of Sigma Chemical Co. Butylated hydroxytoluene (4-methyl-2,6-di-tert-butylphenol, BHT) was a product of Nutritional Biochemical Co., Cleveland, Ohio. The phosphonic analogue of phosphatidylethanolamine (synthetic dimyristoylglyceryl-aminoethylphosphonic acid) was a gift from Professor E. Baer. Glycerolphosphorylethanolamine and glyceryl-AEP were prepared from the corresponding lipids by mild alkaline hydrolysis (see "Methods" below). Whatman DEAE-cellulose was a product of W. & R. Balston Ltd. Silicic acid for column chromatography was a product of Mallinckrodt Chemical

Works. Silicic acid G for thin layer chromatography was a product of E. Merck A.G. Darmstadt.

All the solvents used for lipid chromatography were purified by distillation and contained 0.005% (w/v) of butylated hydroxytoluene (BHT) as an antioxidant (Wren and Szczepanowska, 1964).

#### B. Methods

Maintenance of *Tetrahymena* stocks and culture conditions. *Tetrahymena* stocks were carried in 5 ml of 2 per cent Difco proteose-peptone, 0.5 per cent Difco yeast extract in upright, screw-top tubes and transfers were made at bi-weekly intervals. The organism was grown axenically at 35° in conical flasks, filled to 0.3 of their nominal capacity with the appropriate medium, and shaken on a "Gyrotory" shaker (New Brunswick Scientific Co.) at 170 oscillations per min. Flasks were inoculated with the stationary-phase suspension from a 5 ml tube, and cells were harvested at appropriate times as described in the text.

Two types of media were used. One, referred to below as the "complex medium", contained 2 per cent proteose-peptone, 0.5 per cent yeast extract, 0.05 per cent Tween-80,  $9 \times 10^{-5}$  M Fe-chelate (Conner and Cline, 1964) and 1 per cent glucose. The first four components were dissolved in water at about 70° and the solution filtered. The flasks were charged with the medium, plugged, and autoclaved at 15 lb for 15 min. Glucose was autoclaved separately as a 10 per cent solution at pH 3 (adjusted with  $H_2SO_4$ ) and added under sterile condition to the correct concentration. An alternative medium, which will be referred to as the "defined medium", was used whenever cells were to be presented with a known concentration of

phosphate. Its composition was essentially as described by Stockstad, Seaman, Davis and Hutner (1956), with some modification (Rosenberg, 1966).

Log-phase cells were harvested from 17-hour-old cultures in the complex medium. Unless otherwise indicated, the "log-phase cells" will be referred to as the cells grown 17 hours after inoculation.

Cells were harvested by low-speed centrifugation (500 g, 5 min.).

Acid-soluble and "residual" fractions of cells were prepared as follows. Centrifuge tubes containing the cell pellets were frozen by dipping in a dry ice-ethanol mixture. Ten volumes of 5% (w/v) trichloroacetic acid were added to the frozen cells and the pellets thoroughly dispersed. After standing 10 min. at room temperature, the suspension was centrifuged. The supernatant was decanted and extracted 3 times with an equal volume of ether to remove trichloroacetic acid. This aqueous solution was referred to as "acid-soluble fraction". The precipitate from 5% trichloroacetic acid was extracted twice with 20 volumes of chloroform/methanol (2:1, v/v). The insoluble residue after solvent extraction was referred to as the "residual" fraction. The chloroform-methanol extract containing the lipid was taken to dryness under reduced pressure, with the external bath temperature kept below 40°. The dried residue was re-extracted with chloroform, and the residual material was discarded. The chloroform-soluble material will be referred to as the "lipid" fraction. In specific cases when lipid analysis was contemplated, trichloroacetic acid was not used. The extraction of lipids in such cases is described in the relevant section.

Saponification (mild alkaline hydrolysis) of the



lipid material was carried out by the methods of Dawson (1960) or, when handling small amounts of lipid, by a modified method (Tarlov and Kennedy, 1965).

Acid hydrolysis designed to mineralize all combined phosphorus, except that bound directly to carbon (phosphonate), was carried out in sealed tubes in 6N HCl at 120° for 48 hours. This will be referred to as "drastic" acid hydrolysis. "Mild" acid hydrolysis, applied in some cases, consisted of heating samples in 4N HCl at 100° for 17 hours. Following acid hydrolysis, the cooled mixture was extracted three times with ether to remove fatty acids etc., filtered through glass paper and taken to dryness under reduced pressure to remove HCl.

Estimation of AEP and of inorganic orthophosphate, derived from ester-phosphate and present in acid hydrolysates, was carried out after complete separation of the two compounds by ion-exchange chromatography of the acid-free residue mentioned above. This simple procedure for separation relied on the fact that, while both AEP and orthophosphate were adsorbed on Dowex-1 x 2 (acetate) resin from an aqueous solution, the former was quantitatively eluted with 5% (v/v) acetic acid, while the latter remained on the resin and was eventually removed with 2N HCl. Comparison of phosphorus content and radioactivity of the two fractions allowed the assay of phosphonate and ester phosphorus in various samples, and the calculation of specific radioactivities.

Electrophoresis on Whatman 3MM paper was carried out at pH 2.0 in a water-cooled plate apparatus (Atfield and Morris, 1961) under a voltage gradient of 100 V per cm. The buffer used contained 12.4 ml of formic acid and 43.5 ml of glacial acetic acid per liter.

Amines and related compounds were located on paper



by dipping the dried chromatograms in 0.2% (w/v) ninhydrin in acetone and then heating for 10 minutes at 80°.

Autoradiographs were prepared by exposing the chromatograms for a suitable time interval to either Kodak "Blue Brand" or Ilford Industrial-G film.

Radioactivity of  $^{32}\text{P}$  was measured with a Nuclear Chicago end window gas-flow counter with a thin mylar window. The efficiency of the counter for  $^{32}\text{P}$  is 40%.

Paper chromatography on Whatman 3MM paper was carried out using the following solvent systems:

I. Water-saturated phenol, acetic acid, ethanol (100:10:12, by vol.).

II. n-Butanol, acetic acid, water (4:1:5, by vol.), upper layer used.

Thin-layer chromatography on silicic acid G, or chromatography on silicic acid-impregnated paper were performed with the following solvent systems:

III. Diisobutylketone, glacial acetic acid, water (40:20:3, by vol.).

IV. Chloroform, methanol, water (65:25:4, by vol.). Solvent systems III and IV contained 0.1% butylated hydroxytoluene as an antioxidant (Wren and Szczepanowska, 1964).

Lipids were detected on thin-layer plates and on silicic acid papers by spraying with a 0.001% (w/v) solution of rhodamine 6G in 0.25 M  $\text{K}_2\text{HPO}_4$  followed by ultra-violet light irradiation while the chromatogram was still wet.

Removal of solvents: Unless otherwise stated, solvents (including water and hydrochloric acid or acetic acid) were removed by evaporation under reduced pressure (water pump) in a rotatory evaporator (W. Büchi Scientific Apparatus, Flawil, Switzerland) fitted with a water-cooled

condenser. The rotating flask was heated in a water-bath at various temperatures, but not exceeding 40° for removal of solvents from lipids and up to 70° for the removal of water, hydrochloric acid or acetic acid. The expression "taken to dryness" will be used in short for this procedure.

Total phosphorus was estimated by two methods. When a relatively high amount of phosphorus ( $\sim 10\%$ ) was present, the sample was digested in a mixture of  $\text{H}_2\text{SO}_4$ - $\text{HNO}_3$  on an electric furnace (about 350°) and the heating was continued for 15 min. after the flask contents cleared. This was followed by hydrolysis of pyrophosphate in the flask in 2N acid at 100° for 1 hour. The contents were then neutralized with KOH and the orthophosphate was determined by King's (1932) method. With samples of low phosphorus content ( $\sim 4\%$ ), Bartlett's (1959) method was employed.

Sugars as well as glycerol were detected on paper chromatograms according to the method of Trevelyan, Procter and Harrison (1950), with the following modification:

Reagent

1.  $\text{AgNO}_3$  in acetone : To one drop (0.03 ml) of saturated aqueous silver nitrate solution was added 20 ml of acetone, followed by water dropwise with stirring, until the silver nitrate dissolved.
2. 0.2N alcoholic NaOH solution : Saturated aqueous NaOH solution (approx. 20N) was diluted with ethanol and made to 0.2N.

The chromatogram was sprayed with the two reagents, in the order shown.

Glycerol was estimated by a procedure described by Hanahan and Olley (1958). The recovery of glycerol after acid hydrolysis and removal of acid by evaporation under reduced pressure was found to be 90%.

DEAE-cellulose columns (2 cm x 12 cm) were prepared

according to Rouser et al. (1963), and finally equilibrated with chloroform/methanol (9:1, v/v). Silicic acid columns (1.5 cm x 8 cm) were prepared according to Marinetti, Erbland and Kochen (1957).

Thin-layer silicic acid plates were prepared by applying silicic acid G to glass plates (20 cm x 20 cm) with the aid of a Stahl Applicator (Mangold, 1961).

All procedures involving the handling of lipid fractions (including solvent removal) were carried out under nitrogen.

### 3. Results

#### A. The pattern of AEP labelling in Tetrahymena

##### i. Incorporation of [ $^{32}$ P]orthophosphate into [ $^{32}$ P]AEP in Tetrahymena fractions.

The occurrence of AEP in Tetrahymena was first reported by Kandatsu and Horiguchi (1962) who obtained crystalline AEP from hydrolysates of Tetrahymena cells. The labelling pattern (see Introduction) suggested that in Tetrahymena, free AEP was a breakdown product of macromolecules and that it was not synthesized in the free form.

In order to gain further information about phosphonate bond formation in Tetrahymena, an experiment similar to that described by Rosenberg (1964) involving [ $^{32}$ P]orthophosphate incorporation into [ $^{32}$ P]AEP was carried out, except that the cells were re-suspended in fresh medium containing [ $^{32}$ P]orthophosphate and the incubation times were reduced.

It was found (Fig. II.1) that the bulk of the [ $^{32}$ P]AEP synthesized over the 1.5 hour incubation period was present in the lipid fraction. The rate of incorporation of [ $^{32}$ P]orthophosphate into [ $^{32}$ P]AEP was also highest in the

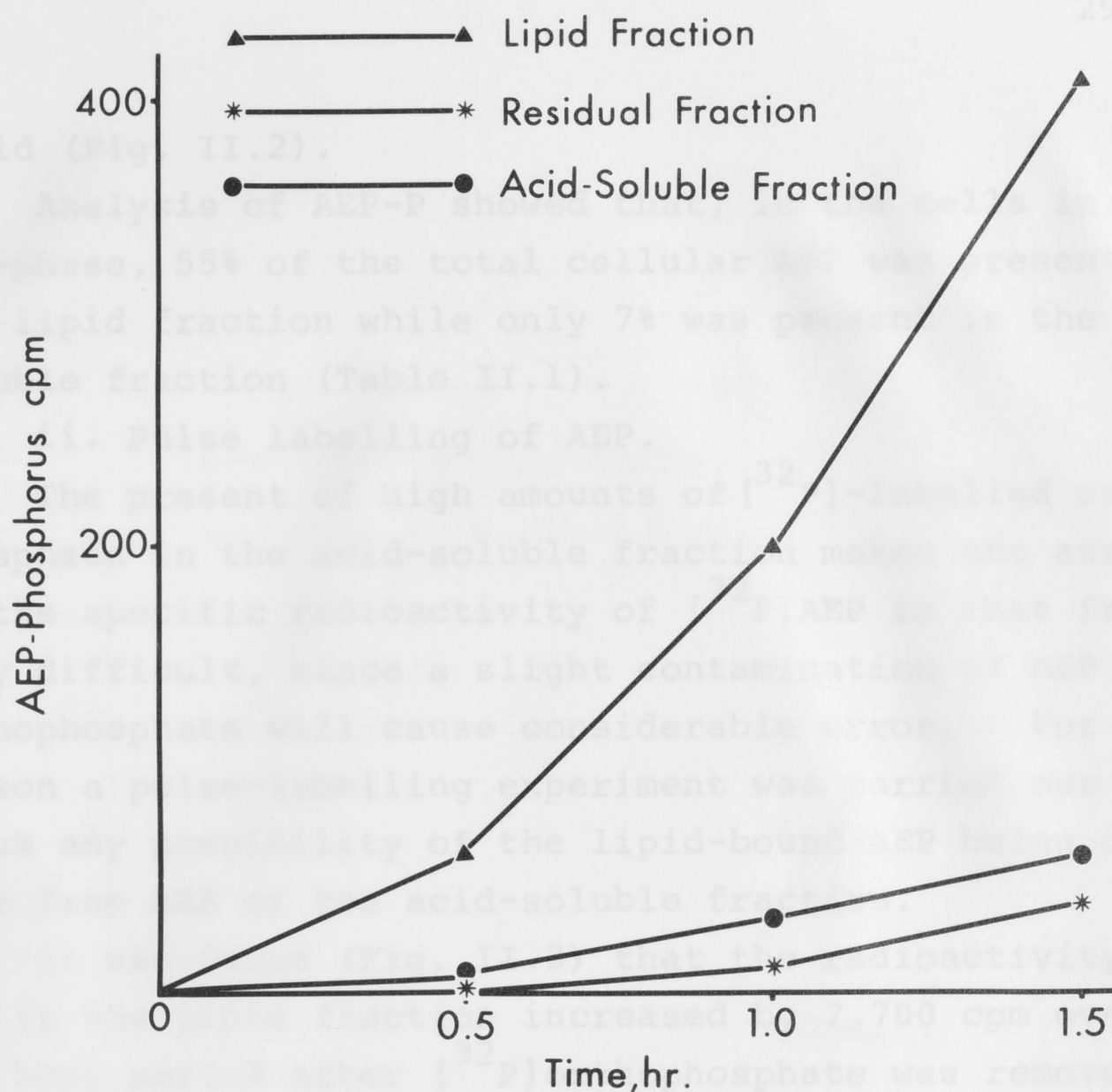


Fig. II.1. Incorporation of [ $^{32}$ P]orthophosphate into [ $^{32}$ P]AEP in fractions of Tetrahymena cells.

Tetrahymena were grown in 600 ml of the "complex medium" at 35° for 17 h. and 5 g. of the wet packed cells were harvested. They were suspended in 70 ml of the "defined medium" containing 0.5 mM and 5  $\mu$ C of [ $^{32}$ P]orthophosphate. The suspension was incubated at 35° and 10 ml samples were withdrawn at intervals, cooled on ice and centrifuged. The cells were washed once with cold saline. Acid-soluble, lipid and residual fractions of the cells were prepared as described above. After "drastic" hydrolysis, [ $^{32}$ P]AEP was separated from [ $^{32}$ P]orthophosphate and its radioactivity was assayed (cf. "Methods").



lipid (Fig. II.2).

Analysis of AEP-P showed that, in the cells in the log-phase, 55% of the total cellular AEP was present in the lipid fraction while only 7% was present in the acid soluble fraction (Table II.1).

ii. Pulse labelling of AEP.

The presence of high amounts of [ $^{32}\text{P}$ ]-labelled orthophosphate in the acid-soluble fraction makes the assay of the specific radioactivity of [ $^{32}\text{P}$ ]AEP in that fraction very difficult, since a slight contamination of AEP with orthophosphate will cause considerable error. For that reason a pulse-labelling experiment was carried out to check any possibility of the lipid-bound AEP being derived from free AEP of the acid-soluble fraction.

It was found (Fig. II.3) that the radioactivity of AEP in the lipid fraction increased by 7,700 cpm over a two hour period after [ $^{32}\text{P}$ ]orthophosphate was removed from the medium. The radioactivity of AEP in the acid-soluble fraction did not change appreciably in this time. Furthermore, following a short time of incubation of the cells with [ $^{32}\text{P}$ ]orthophosphate (1 h.), the radioactivity of AEP in the acid-soluble fraction was only 3% of that in lipid-bound AEP. These facts indicate that free AEP cannot be the precursor of the bulk of the lipid-bound AEP. They also make free AEP an unlikely site of phosphonate (C-P) bond biosynthesis.

iii. Specific radioactivities of AEP and esterphosphate in Tetrahymena lipid.

The results from previous experiments suggested that "phosphonolipid" (term suggested by Baer and Stanacev, 1964) might be the site of formation of the phosphonate bond in Tetrahymena. In order to obtain further information about the biosynthesis of the phosphonate bond as

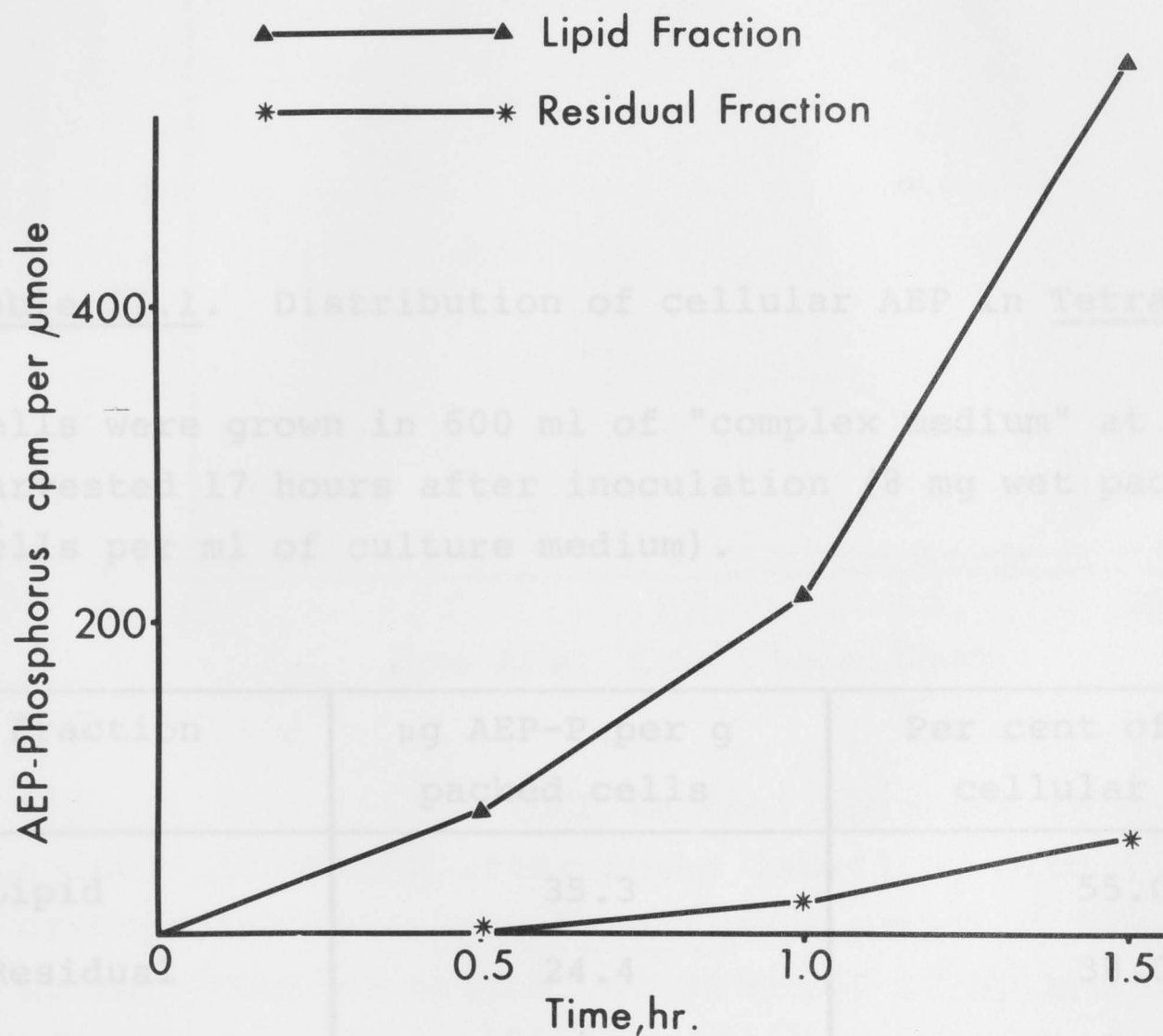


Fig. II.2. Specific radioactivity of [ $^{32}\text{P}$ ]AEP in cell fractions of Tetrahymena incubated in a medium containing [ $^{32}\text{P}$ ]orthophosphate. (For experimental conditions, see Fig. II.1).

Table II.1. Distribution of cellular AEP in Tetrahymena.

Cells were grown in 600 ml of "complex medium" at 35° and harvested 17 hours after inoculation (8 mg wet packed cells per ml of culture medium).

Fraction	μg AEP-P per g packed cells	Per cent of total cellular AEP
Lipid	35.3	55.0
Residual	24.4	38.0
Acid-soluble	4.5	7.0

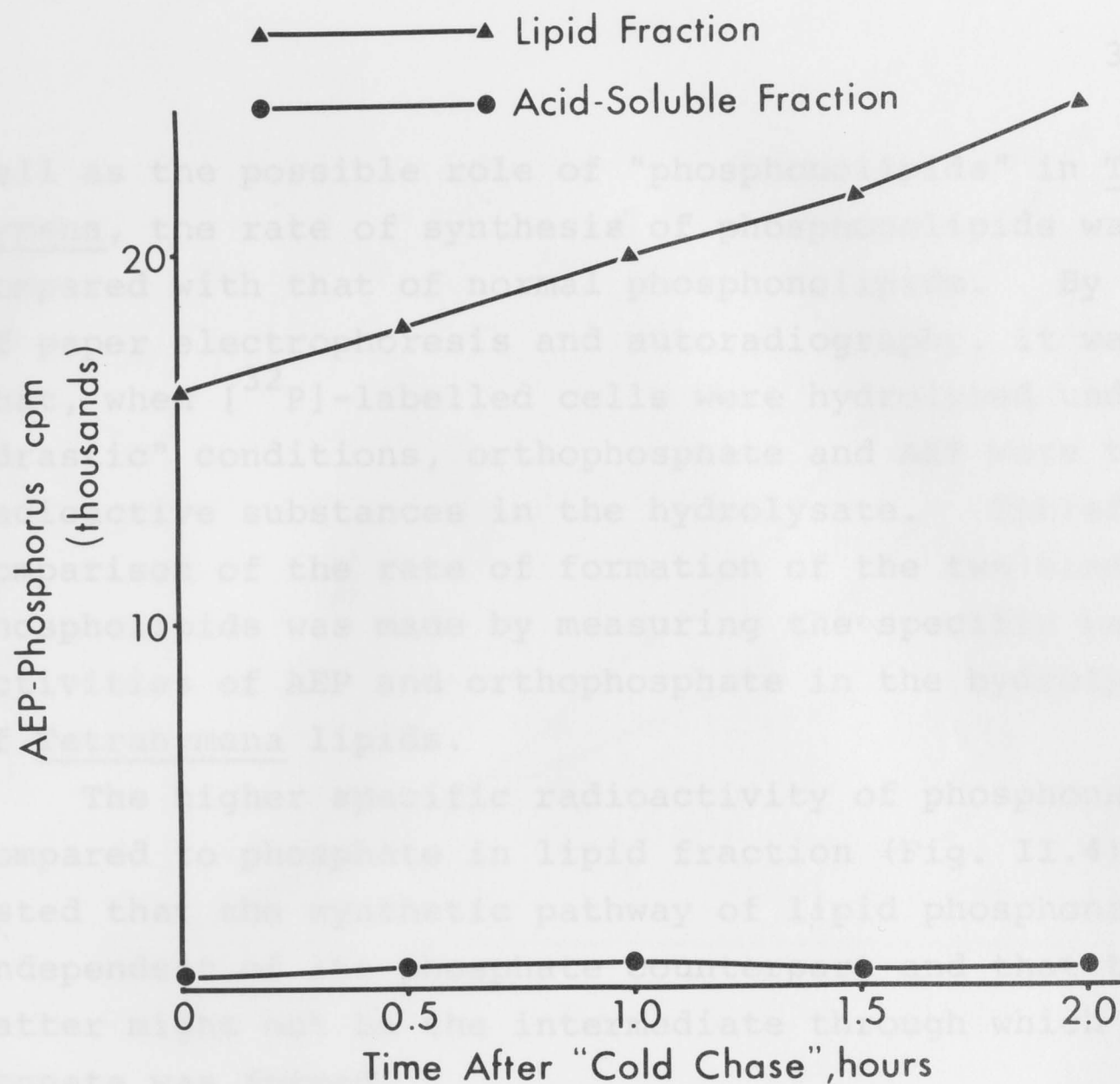


Fig. II.3. [ $^{32}\text{P}$ ]AEP in the lipid and acid-soluble fractions after pulse labelling with [ $^{32}\text{P}$ ]-orthophosphate. Tetrahymena were grown in 300 ml of the "complex medium" at 35°C for 17 h. and 2.5 g of the wet packed cells were harvested. The cells were suspended in 70 ml of the "defined medium" containing 0.3 mM and 50  $\mu\text{C}$  of [ $^{32}\text{P}$ ]orthophosphate. The suspension was shaken at 35° for 1 h. The cells were collected by centrifugation and washed twice with saline. The [ $^{32}\text{P}$ ]-labelled cells were resuspended in 70 ml of freshly prepared "defined medium" containing 10 mM "cold" orthophosphate. The suspension was again shaken at 35° and 10 ml samples were pipetted out at intervals for the assay of [ $^{32}\text{P}$ ]AEP in the cells. After the hydro-



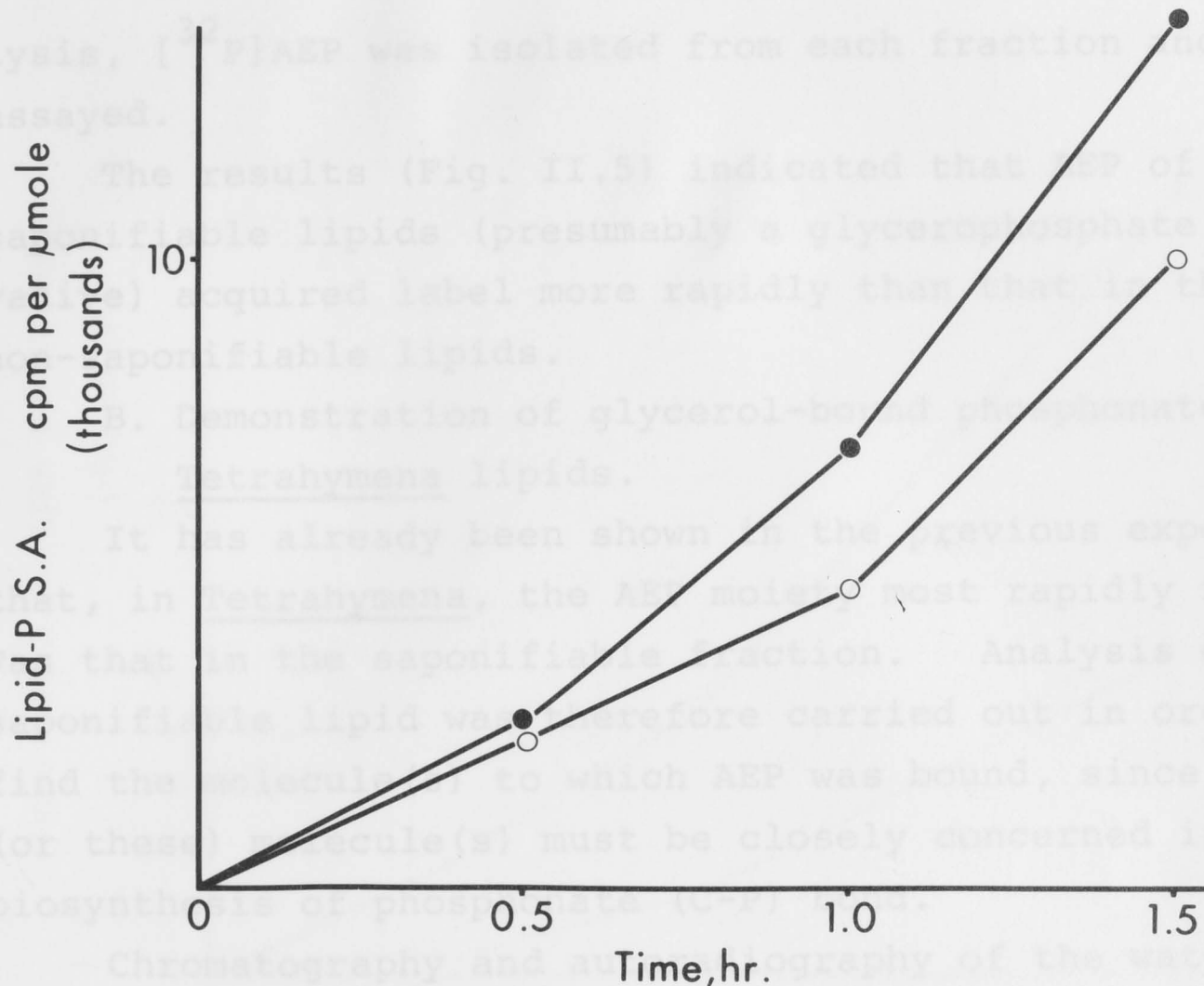
well as the possible role of "phosphonolipids" in Tetrahymena, the rate of synthesis of phosphonolipids was compared with that of normal phosphonolipids. By means of paper electrophoresis and autoradiography, it was shown that, when [ $^{32}\text{P}$ ]-labelled cells were hydrolysed under "drastic" conditions, orthophosphate and AEP were the only radioactive substances in the hydrolysate. Therefore the comparison of the rate of formation of the two kinds of phospholipids was made by measuring the specific radioactivities of AEP and orthophosphate in the hydrolysate of Tetrahymena lipids.

The higher specific radioactivity of phosphonate compared to phosphate in lipid fraction (Fig. II.4) suggested that the synthetic pathway of lipid phosphonate is independent of its phosphate counterpart and that the latter might not be the intermediate through which phosphonate was formed.

iv. Specific radioactivities of phosphonate (AEP) in saponifiable and non-saponifiable lipids of Tetrahymena.

In the hope of gaining further information on the site of phosphonate bond biosynthesis, the specific radioactivities of AEP in saponifiable and non-saponifiable lipid fractions were compared.

The experiment was carried out in the same way as those described above. [ $^{32}\text{P}$ ]-Labelled Tetrahymena lipid was subjected to mild alkaline hydrolysis (saponification) (cf. "Methods"). After saponification of the lipids, the aqueous phase (saponifiable) was subjected to "drastic" hydrolysis directly, while the "lipid" phase (non-saponifiable, remaining in the solvent) was taken to dryness to remove solvent. The residue was suspended in 6N HCl and subjected to "drastic" hydrolysis. After the hydro-



**Fig. II.4.** Specific radioactivities of lipid-bound phosphate and AEP in Tetrahymena cells. Log phase cells grown in 300 ml of the "complex medium" were harvested and the pellet (2.5 ml) was suspended in 70 ml of the "defined medium" containing 1 mM of [ $^{32}$ P]orthophosphate (2  $\mu$ C). The cell suspension was incubated at 35° in the same way as in the previous experiments and 10-ml samples were withdrawn at intervals. The cell lipid was extracted and, after drastic hydrolysis, the specific radioactivity of the AEP and orthophosphate (from ester-phosphate) released was determined.

- — ○ Specific radioactivity of ester-phosphate
- — ● Specific radioactivity of AEP

lysis, [ $^{32}\text{P}$ ]AEP was isolated from each fraction and assayed.

The results (Fig. II.5) indicated that AEP of the saponifiable lipids (presumably a glycerophosphate derivative) acquired label more rapidly than that in the non-saponifiable lipids.

B. Demonstration of glycerol-bound phosphonate in Tetrahymena lipids.

It has already been shown in the previous experiments that, in Tetrahymena, the AEP moiety most rapidly formed was that in the saponifiable fraction. Analysis of saponifiable lipid was therefore carried out in order to find the molecule(s) to which AEP was bound, since this (or these) molecule(s) must be closely concerned in the biosynthesis of phosphonate (C-P) bond.

Chromatography and autoradiography of the water-soluble compounds obtained after saponification of Tetrahymena lipids showed (Fig. II.6) the presence of at least four major radioactive phosphorus-containing compounds.

The corresponding areas on the paper were cut out and eluted with water. A portion of each fraction was hydrolysed under "drastic" condition and the resulting orthophosphate and AEP (if any) were separated by passage through Dowex-1 (acetate) as described in "Methods". Table II.2 shows that the fraction designated "C" contained the major proportion of the phosphonate (as well as ester phosphate) phosphorus.

The remainder of fraction C was then applied to a column (1.5 cm x 3 cm) of Dowex-1 x 2 (acetate). The entire radioactive material was found to emerge with the water front. Further elution with either 5% acetic acid or 2N HCl failed to show any radioactivity. The water effluent was collected, concentrated under reduced pres-

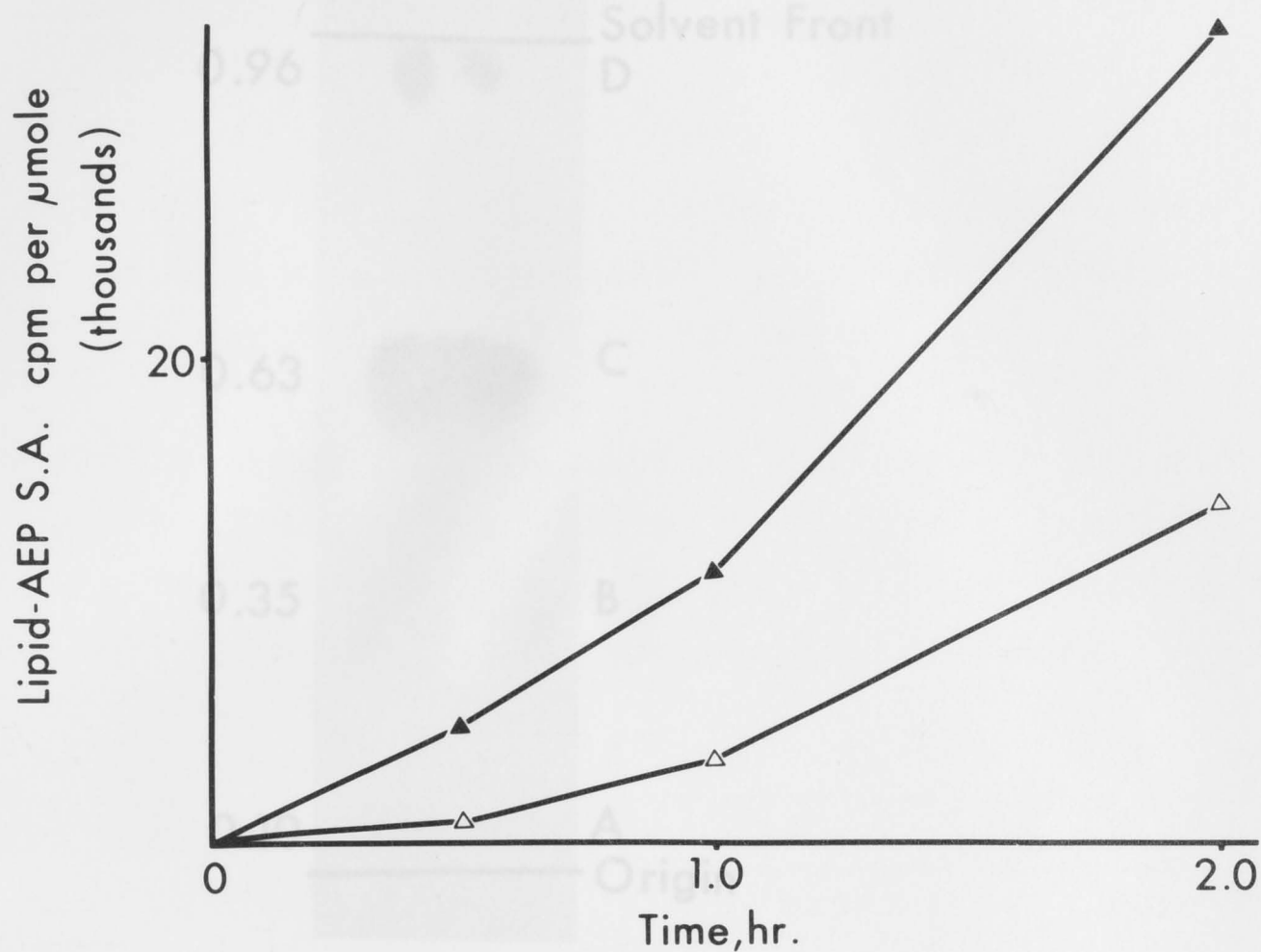


Fig. II.5. Specific radioactivity of [<sup>32</sup>P]AEP in the saponifiable and non-saponifiable lipid fractions of Tetrahymena. Experimental conditions were as in Figure II.4. After the cell lipid was extracted, it was subjected to mild alkaline hydrolysis and the specific radioactivity of [<sup>32</sup>P]-AEP in the saponifiable and non-saponifiable fractions was assayed.

▲ — ▲ [<sup>32</sup>P]AEP in saponifiable lipid fraction  
 △ — △ [<sup>32</sup>P]AEP in non-saponifiable lipid fraction



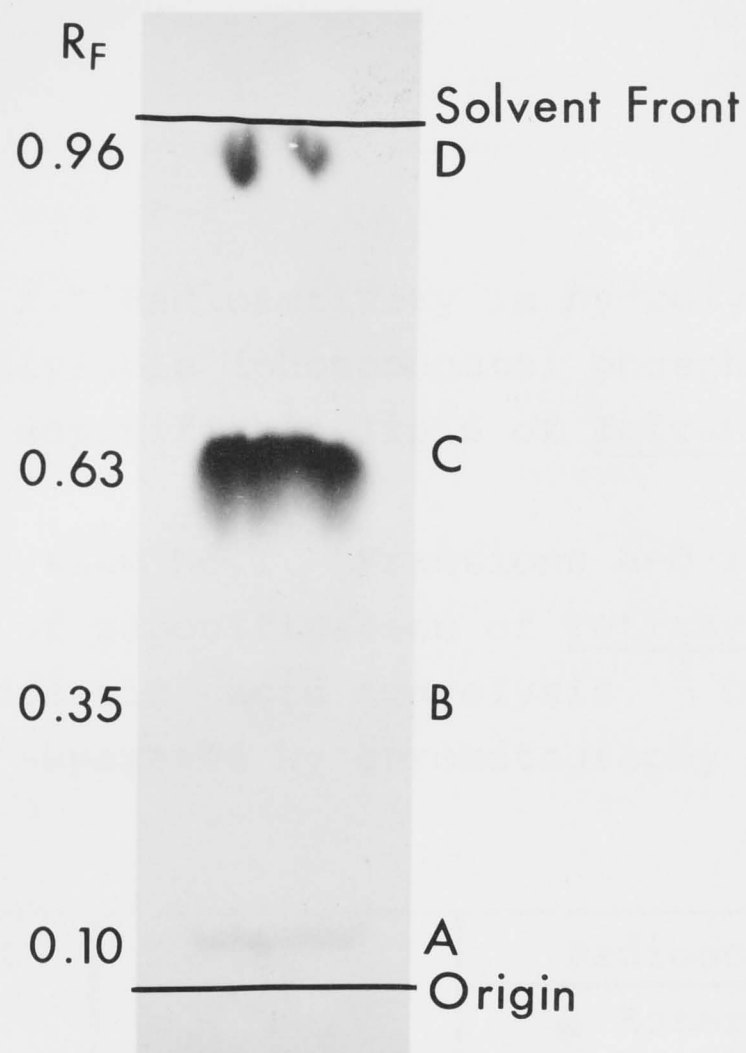


Fig. II.6. Autoradiography of a paper chromatogram of the saponified lipid from Tetrahymena. The cells were grown in 300 ml of the "complex medium" at 35° for 17 h. and 2.8 g of the wet packed cells were harvested. They were suspended in 50 ml of the "defined medium" containing 0.2 mM and 10  $\mu$ C of [ $^{32}$ P]orthophosphate. The cell suspension was incubated in a rotatory shaker at 35° for 2 h. The [ $^{32}$ P]-labelled cells were centrifuged off and washed with saline. The lipid was extracted from the packed cells and saponified. The water-soluble layer of saponified lipid was separated by centrifugation and was taken to dryness. The residue was taken up in a small amount of water and applied to Whatman 3 MM chromatographic paper. It was developed (ascending direction) with solvent system I for 17 h. The dried chromatogram was exposed to X-ray film overnight.

Table II.2. Radioactivity in hydrolysable (ester) and non-hydrolysable (phosphonate) phosphorus components of the saponifiable lipid of Tetrahymena.

Solvent system No.I. Fractions A-D represent radioactive products of saponification of Tetrahymena lipids, subjected to "drastic" acid hydrolysis. Orthophosphate and AEP were separated by chromatography on Dowex-1 acetate.

Fraction	$R_F$	Radioactivity (counts/min)	
		Ester P	Phosphonate
A	0.10	2600	91
B	0.35	1212	89
C	0.63	47400	4990
D	0.96	10250	112

sure and applied to a column (1.5 cm x 3 cm) of Dowex-50 ( $H^+$  form), when the radioactive material again emerged on washing with water. No further radioactivity could be recovered from the column by passage of 2.5N  $NH_4OH$ . The fraction was then subjected to paper chromatography in solvent systems I and II, and to paper electrophoresis at pH 2.0 (see "Methods"). The papers were developed with ninhydrin and autoradiographs were prepared. In all cases, one spot only could be seen, the radioactivity corresponding exactly to the ninhydrin-stained area. The spots had  $R_F$  values of 0.63 and 0.15, respectively, in solvent systems I and II. Under the conditions employed for electrophoresis, the compound remained at the origin.

Since the  $R_F$  values obtained for the unknown were the same as quoted (Dawson, 1960) for glycerylphosphoryl-ethanolamine in the solvent system I, attempts were made to demonstrate the presence of a glycerol moiety in the fraction. Samples of fraction C were subjected to either "mild" or "drastic" hydrolysis (see "Methods") and the hydrolysates were rendered free of acid and fractionated as shown in Fig. II.7.

Fraction G (Fig. II.7) from both the "mild" and "drastic" hydrolysates, which was expected to contain any free glycerol present, was concentrated and subjected to paper chromatography in solvent system II, together with authentic glycerol as a reference. The dried paper was treated with the alkaline silver reagent (cf. "Methods") which showed that only one reacting compound was present in each hydrolysate (Fig. II.8). The compound moved with an  $R_F$  of 0.52, which was the same as that of authentic glycerol.

In solvent system II, authentic mannose and inositol gave  $R_F$  values of 0.26 and 0.13, respectively, and could

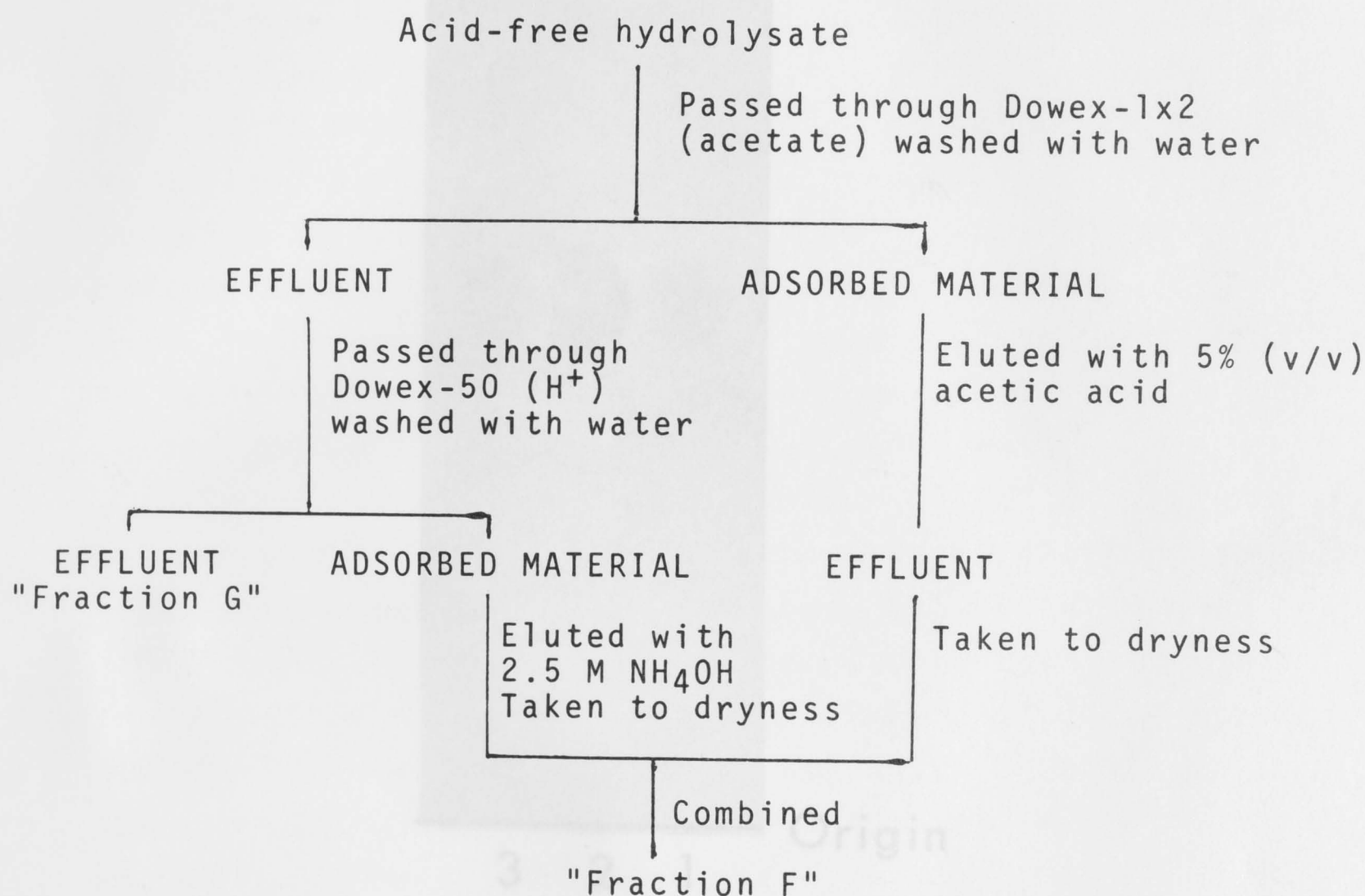


Fig. II.7. Fractionation of products of "mild" and "drastic" hydrolysis of "fraction C" (Table II.2) from saponified lipids.

1. Fraction G (Fig. II.7) from mild hydrolysate
2. Authentic glycerol
3. Fraction G (Fig. II.7) from drastic hydrolysate.



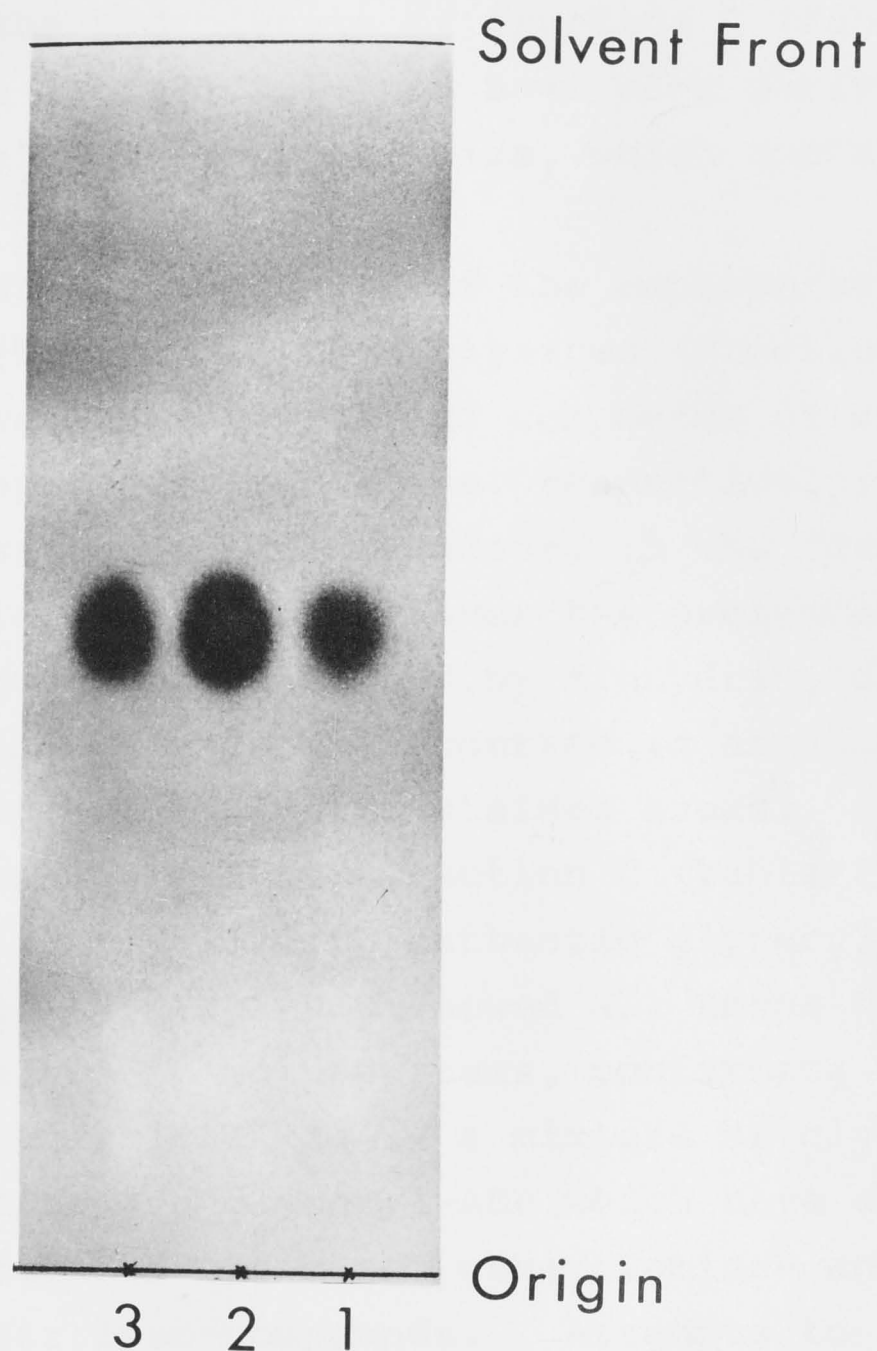


Fig. II.8. Paper chromatogram of "fraction C" (Table II.2) hydrolysate, after spraying with alkaline silver nitrate reagent.

1. Fraction G (Fig. II.7) from mild hydrolysate
2. Authentic glycerol
3. Fraction G (Fig. II.7) from drastic hydrolysate.

be separated from glycerol. The absence of mannose and inositol from the hydrolysate of fraction C (Table II.2) showed that the latter could not have been derived from the breakdown of mannophospholipids, which contain mannose and inositol.

Electrophoresis at pH 2.0 of the amphoteric components of fraction C (Table II.2) hydrolysates (Fraction F, Fig. II.7) showed the presence of compounds which migrated as AEP and phosphorylethanolamine, respectively, in the "mild" hydrolysate, and of AEP alone, in the "drastic" hydrolysate (Fig. II.9). Wherever the presence of these two compounds was detected by ninhydrin, darkening of the X-ray film on exposure occurred in areas precisely corresponding to the ninhydrin-stained areas.

Paper chromatography of fraction C (Table II.2) in solvent systems I and II with authentic glycerylphosphorylethanolamine and glyceryl-AEP showed all three to have identical  $R_F$  values in both systems, confirming the fact that fraction C (Table II.2) is a mixture of glycerylphosphorylethanolamine and glyceryl-AEP which have arisen in the saponification of the mixed ethanolamine- and AEP-cephalin or their lyso-compounds. Attempts to separate glycerylphosphorylethanolamine from glyceryl-AEP by ion-exchange chromatography or paper chromatography have so far been unsuccessful.

Analysis of a sample of fraction C (Table II.2) afforded the values 4.98 and 4.72  $\mu$ moles for total phosphorus and glycerol, respectively, giving a P/glycerol value of 1.05. The ratio of phosphate-P to phosphonate-P was found to be 13.

The specific radioactivities of glyceryl-AEP and glyceryl-phosphorylethanolamine in fraction C (Table II.2) were also compared.

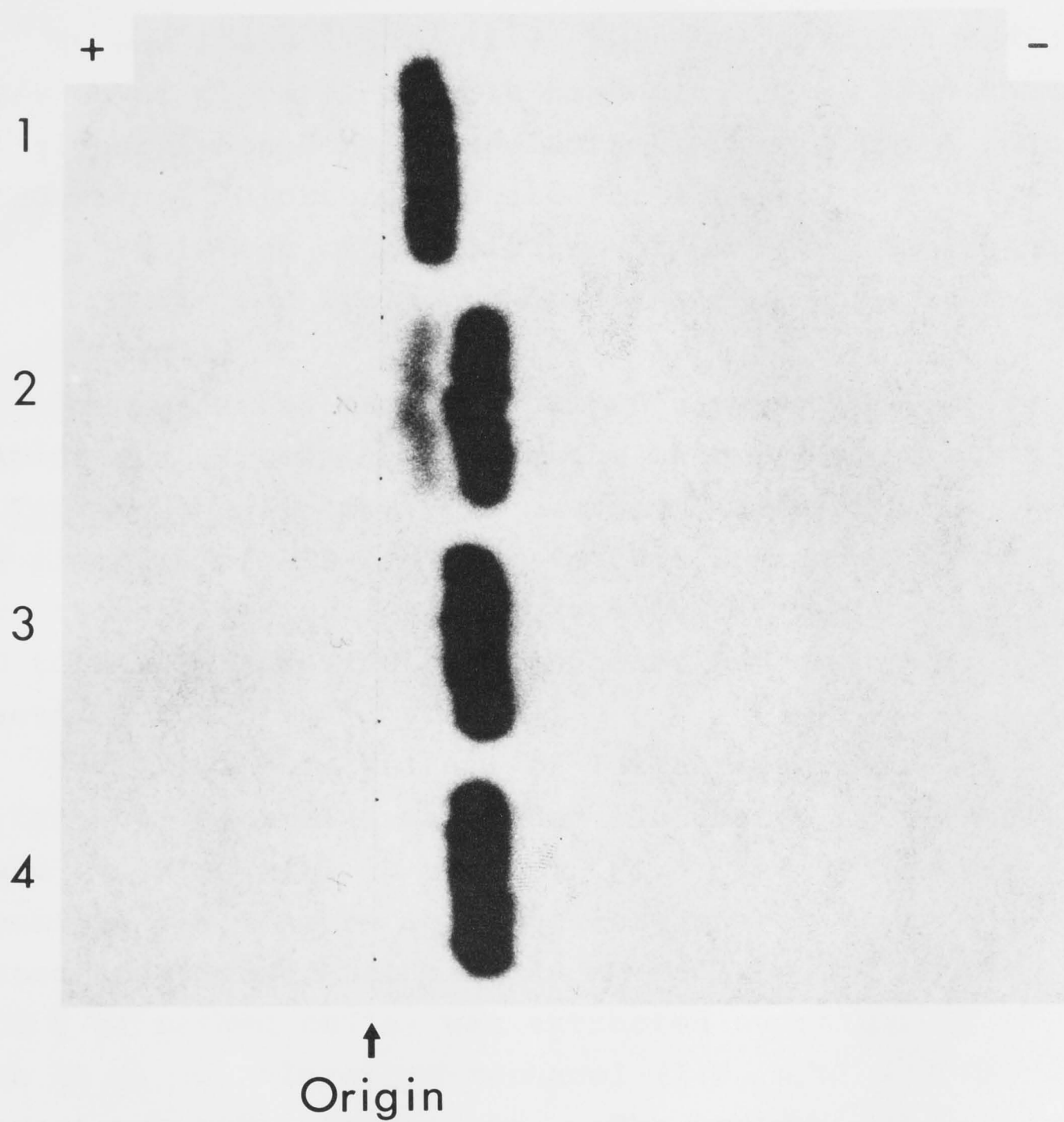


Fig. II.9. Paper electrophoresis of "fraction C" (Table II.2) hydrolysates. The paper was stained with ninhydrin.

1. Authentic phosphorylethanolamine
2. Mild hydrolysate
3. Drastic hydrolysate
4. Authentic AEP

It was found (Table II.3) that the specific radioactivity of glyceryl-AEP was somewhat higher than that of the glycerylphosphorylethanolamine in Tetrahymena incubated with [ $^{32}$ P]orthophosphate for 1 hour.

C. Isolation of the mixture of phosphatidylethanolamine and its phosphonic analogue from Tetrahymena lipid.

The isolation of a mixture of glycerylphosphorylethanolamine and glyceryl-AEP from the saponification products of Tetrahymena lipid in the previous experiment suggested the presence of AEP-cephalin in this species. It was then thought desirable to isolate cephalin from Tetrahymena and to demonstrate the occurrence of AEP-cephalin in that fraction.

To a log phase culture of Tetrahymena cells in 300 ml of the complex medium was added 100  $\mu$ moles of orthophosphate labelled with 10  $\mu$ C of [ $^{32}$ P]. The cells were further incubated for 4 hours on a rotatory shaker at 35°, harvested and washed once with 40 mM NaCl. The pellet (2.6 g of packed cells) was extracted overnight at 2° with 40 ml of chloroform-methanol (1:2, v/v) and the extract was taken to dryness. The residue was extracted with 20 ml of chloroform and the solvent was again removed. The total lipid (70 mg) was dissolved in 3 ml of chloroform-methanol (9:1, v/v) and the solution was applied to a column (2 cm x 12 cm) of DEAE-cellulose in equilibrium with the same solvent. The column was eluted with chloroform-methanol mixtures (v/v) : 9:1 (200 ml), 7:3 (200 ml), and with 150 ml methanol. The effluent was collected in 10 ml fractions. The radioactive material emerged in several peaks (Fig. II.10A).

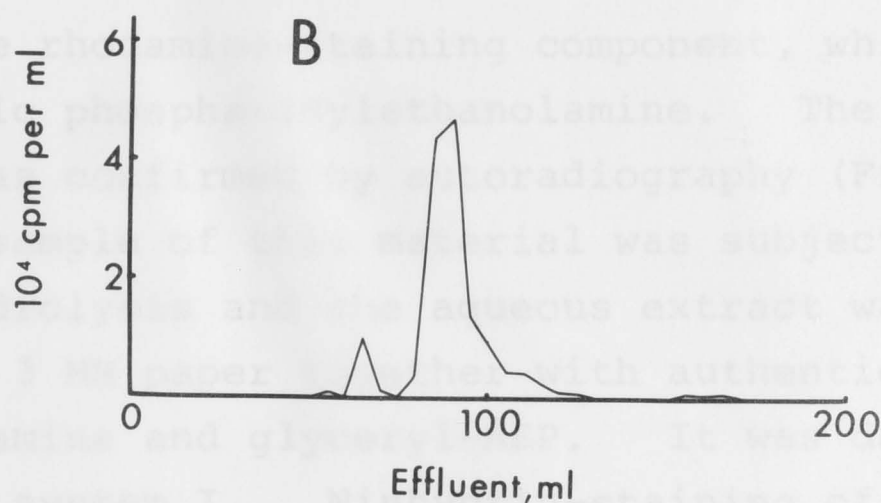
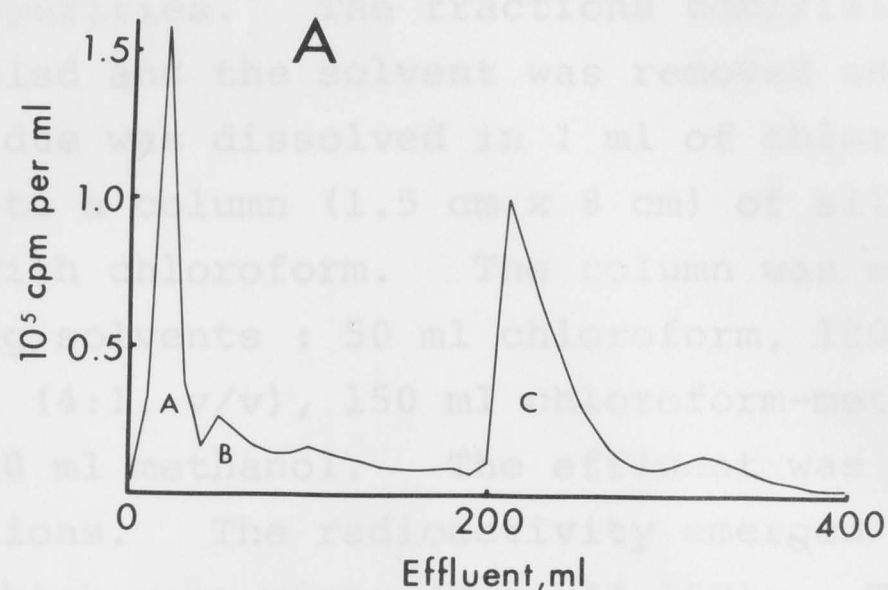
Thin-layer chromatography showed that peak C (Fig. II.10A) contained the phosphatidylethanolamine and some



Table II.3. Comparison of the specific radioactivities of glyceryl-AEP and glycerylphosphorylethanolamine

The mixture of these two compounds was obtained from saponified Tetrahymena lipids. The cells had been incubated for 1 hour in "defined medium" contained 20  $\mu$ C of [ $^{32}$ P]orthophosphate.

	[ $^{32}$ P]-glyceryl-AEP	[ $^{32}$ P]-glycerylphosphoryl-ethanolamine
Amount ( $\mu$ moles per g packed cells)	0.3	4.0
Specific radioactivity (cpm per $\mu$ mole)	31,500	27,800



**Fig. II.10.** Chromatography of the crude lipid from Tetrahymena which had been labelled in vivo with  $^{32}\text{P}$ .

(A) First chromatography on a DEAE-cellulose column.

(B) Re-chromatography, on silicic acid, of the second major peak from the first column (peak (C), upper plot).

Abscissa, effluent volume (ml), ordinate, count/min. per ml effluent.

minor impurities. The fractions comprising the peak were pooled and the solvent was removed under nitrogen. The residue was dissolved in 1 ml of chloroform and applied to a column (1.5 cm x 8 cm) of silicic acid equilibrated with chloroform. The column was eluted with the following solvents : 50 ml chloroform, 100 ml chloroform-methanol (4:1, v/v), 150 ml chloroform-methanol (1:1, v/v), 150 ml methanol. The effluent was collected in 5 ml fractions. The radioactivity emerged in three peaks, two of which were minor (Fig. II.10B). The major peak was checked by thin-layer chromatography in solvent systems III and IV (cf. "Methods") and found to contain only one rhodamine-staining component, which migrated as authentic phosphatidylethanolamine. The position of the spots was confirmed by autoradiography (Fig. II.11).

A sample of this material was subjected to mild alkaline hydrolysis and the aqueous extract was applied to Whatman 3 MM paper together with authentic glycerophosphorylethanolamine and glyceryl-AEP. It was developed with solvent system I. Ninhydrin-staining of the chromatogram showed all three substances to have identical  $R_F$  values (0.63). Autoradiography of the chromatogram showed that only one radioactive spot was detected, the position of which corresponded with the ninhydrin-stained area of the sample.

Portions of the fraction (the major peak, Fig. II.10B) were subjected to "mild" and "drastic" acid hydrolysis and the phosphorus components of the hydrolysates were recovered by passage through Dowex-1 (acetate) (cf. "Methods"). Paper electrophoresis of the hydrolysis products followed by autoradiography showed AEP and orthophosphate to be the products of "drastic" hydrolysis; AEP, phosphorylethanol-

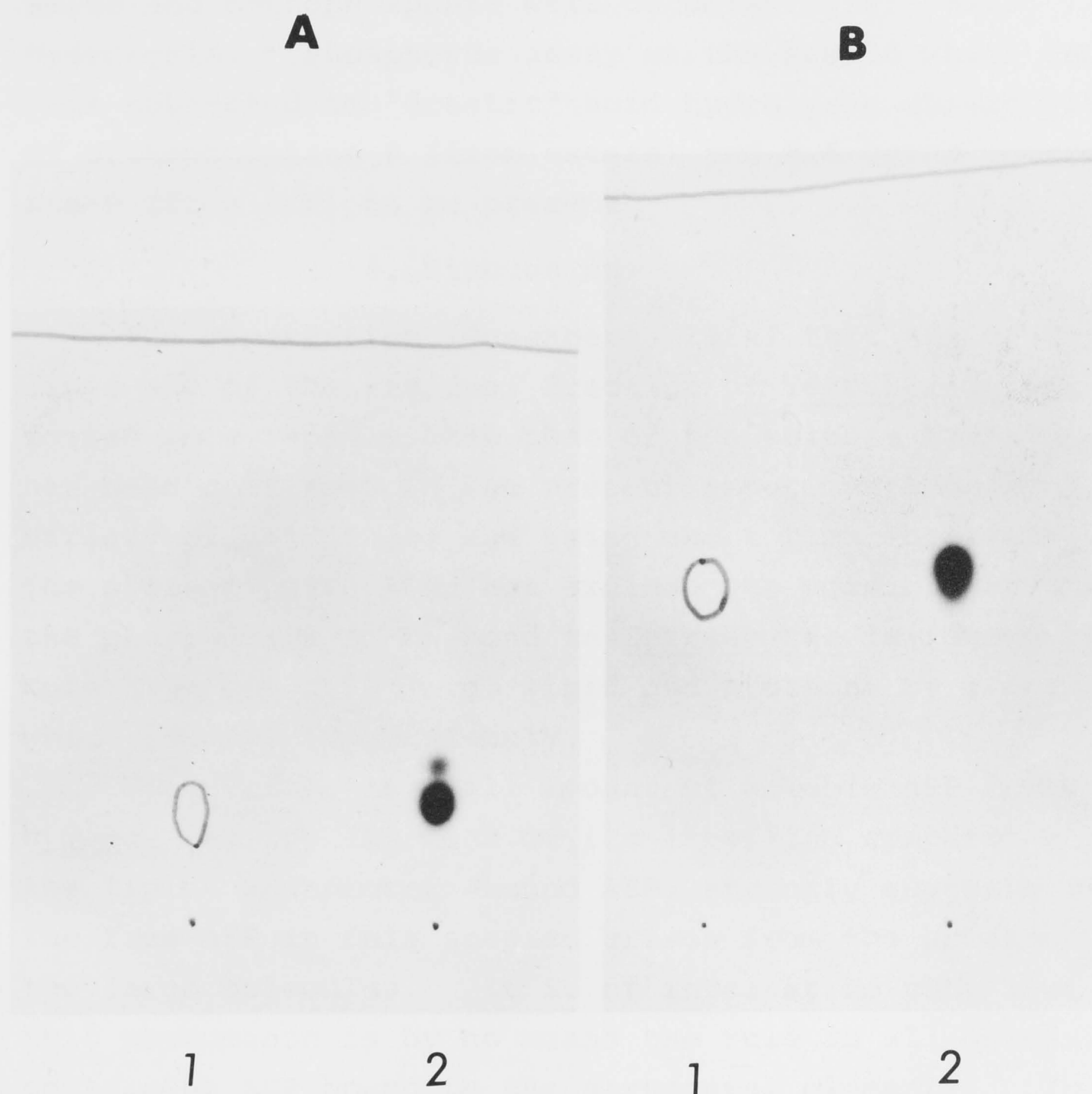


Fig. II.11. Autoradiographs of thin-layer chromatograms of the lipid from the major peak emergent from the silicic acid column (Fig. II.10B). (A) and (B), plates developed in solvent systems III and IV respectively. (1) authentic phosphatidylethanolamine, visualised with rhodamine 6G (the outline of the spot was traced on the film), (2) the isolated lipid.



amine and orthophosphate were detected after "mild" hydrolysis. Phosphorus assay on the sample which had been subjected to "drastic" acid hydrolysis showed 118  $\mu\text{g}$  of orthophosphate-P (from esters) and 8.6  $\mu\text{g}$  of phosphonate-P (from AEP) to be present.

#### 4. Discussion

The observation (Rosenberg, 1964) that AEP of the lipid and of the residual fraction of Tetrahymena was formed more rapidly than that of the soluble fraction, has been confirmed in the present experiments under a variety of conditions and using short time intervals. The present results do not exclude the possibility that the phosphonate (C-P) bond in Tetrahymena is formed at more than one site (e.g. lipid and protein) by processes which operate independently.

The finding of small amount of soluble AEP in Tetrahymena, and the low rate of its labelling compared with the lipid- and protein-bound AEP, strongly suggests that the free AEP in this species arises from the breakdown of the large molecules. It is of interest to note that this phenomenon is by no means the rule in all species containing AEP bound to the structural elements. Thus, although residue- and lipid-bound AEP was found in other species (Quin, 1965; De Koning, 1966; Rouser et al., 1963), the labelling pattern in molluscs (see Chapter V, this thesis) is quite different and suggestive of a soluble intermediate.

While the pattern of AEP labelling in Tetrahymena described above definitely excluded soluble AEP as a precursor of the bulk of the lipid-bound AEP, it was still possible to demonstrate the operation of the classical cephalin synthetic pathway, involving AEP. This pathway,

originally described by Kennedy and Weiss (1956), provides for the synthesis of phosphatidylethanolamine from phosphorylethanolamine via a cytidinediphosphate-ethanolamine intermediate. The inclusion of AEP into "phosphonocephalin" by a corresponding intermediate in Tetrahymena is described in Chapter III of this thesis, where these results are also discussed in detail. At this stage it is important to stress that that pathway cannot be of major significance in this species. It is probably responsible only for a minor re-synthesis of phospholipid from the traces of free AEP present, and may well represent a form of salvage mechanism. The experiments described in this chapter, particularly those dealing with pulse-labelling (Fig. II.3), clearly emphasise this point. Thus, when Tetrahymena were shaken with labelled phosphate which was then substituted with unlabelled material, the radioactivity of the lipid-bound AEP in the cells continued to rise, while that of the soluble AEP did not fall, and was, in fact, many times lower than that of the former.

The occurrence of lipid-bound AEP in a variety of species has now been well established (Horiguchi and Kandatsu, 1959; Rouser et al., 1963; Hori et al., 1964; Rosenberg, 1964; Hori et al., 1966; De Koning, 1966; Kittredge et al., 1962). In most cases AEP has been demonstrated amongst the products of drastic hydrolysis of the tissue lipids or of whole tissues. In the case of the sea anemone, an AEP-containing sphingolipid was isolated (Rouser et al., 1963). In the experiments described in this chapter, AEP of the highest specific radioactivity was found in the saponifiable lipid fraction of Tetrahymena and this clearly indicates the importance of "phosphonocephalin" in C-P bond biosynthesis. It is also noteworthy that the specific radioactivity of the AEP-phos-

phorus in the phosphonocephalin fraction was persistently higher than that of the phosphatidylethanolamine-phosphorus. This renders highly unlikely the suggested (Segal, 1965) route of phosphonate bond synthesis from phosphatidylethanolamine. Further proof against such a possibility rests in the results on the incorporation of [ $^{14}\text{C}$ ]serine into the two fractions (see Chapter IV).

The demonstration of "phosphonocephalin" in Tetrahymena, described in this chapter, is the first authentic demonstration of a naturally occurring product of this nature, although several other reports to this effect exist in the literature. Thus, Kittredge et al. (1962) claimed to have isolated the glycerol ester of AEP (glyceryl-AEP) from the alkaline hydrolysate of the lipids of the sea anemone. However, the compound isolated by these authors proved to be unstable and it was suggested (Horiguchi, 1966) that it may be some other AEP-derivative. In fact, the reported behaviour of the unknown compound on ion-exchange resins was different from that of glyceryl-AEP reported here.

The isolation of AEP-containing cephalins from the house-fly, Musca domestica, was reported by Bridges and Rickets (1966), but the insects had previously been fed AEP. Similarly, Kandatsu et al. (1965) reported that [ $^{32}\text{P}$ ]AEP, when fed to rats, was incorporated into the liver lipids. AEP-containing lipids have also been isolated in small quantities from ruminants (Kandatsu and Horiguchi, 1965; Shimizu et al., 1965). In all those cases AEP was not a natural metabolite of the species investigated, but was either introduced by the experimenter or supplied by the symbiotic microorganism of the rumen. Its incorporation into the lipids of the experimental animals (or hosts) constitutes another instance of

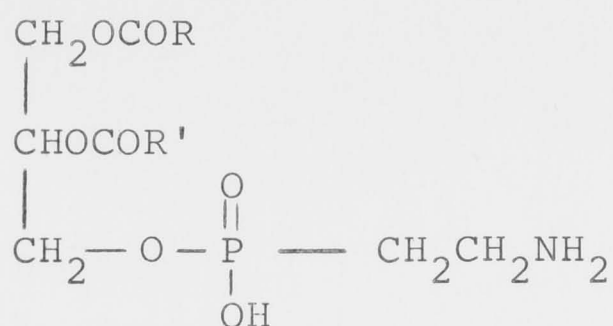


the low specificity which the phospholipid-synthesising enzymes display towards the amino-base (cf. Chojnacki, 1964).

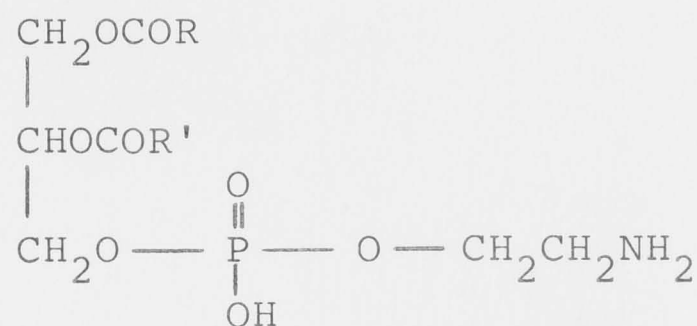
The only mention in the literature of a naturally occurring "phosphonocephalin" was that of Horiguchi (1966), who claimed that such a compound existed in Tetrahymena and that it was the only cephalin present there, to the exclusion of either phosphatidylethanolamine and phosphatidylcholine. Unfortunately, no evidence was offered for either claim, and, while proof for the former has been given in this chapter, Horiguchi's (1966) second claim cannot be reconciled with the clearly proved presence in Tetrahymena of phosphatidylethanolamine, in some thirteen-fold excess of the phosphonic analogue, as reported here.

While the evidence for the presence of "phosphonocephalin" offered in this chapter is quite substantial, it does not include the isolation of a pure phosphonocephalin free from phosphatidylethanolamine. This deficiency is dictated by the fact that the two analogues are very similar in their behaviour on all chromatographic media used, and the separation of corresponding pairs of analogues proved impossible. Thus, all attempts to separate AEP-cephalin from phosphatidylethanolamine (formulae V and VI) or even 1-glyceryl-AEP from 1-glyceryl-phosphorylethanolamine (formulae VII and VIII) were unsuccessful. It was not until these compounds were degraded to the next analogue pair (AEP and phosphorylethanolamine) that separation became possible (cf. "Methods").

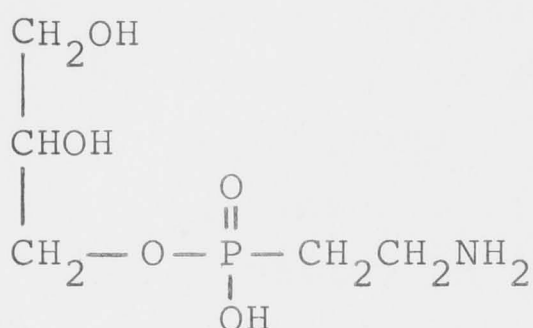




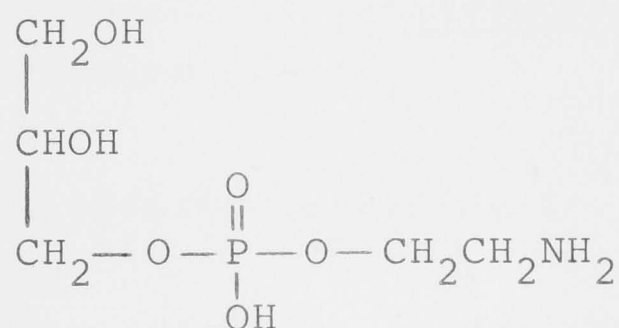
(V)



(VI)



(VII)



(VIII)

In a recent report Baer (1966) indicated that the attempts to separate an artificial mixture of a lecithin and its phosphonic acid analogue by thin-layer chromatography on silica gel H by six solvent systems were unsuccessful. He expressed the hope that, as the properties of the synthetic phosphonolipids are studied more thoroughly, more effective methods for the separation of phosphonolipids from phospholipids will be found.

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CHAPTER III. THE ENZYMIC SYNTHESIS OF THE PHOSPHONIC  
ANALOGUE OF CEPHALIN IN TETRAHYMENA

Summary

1. AEP-cytidyltransferase activity has been shown to be present both in whole Tetrahymena and in cell-free preparations.
2. The reaction catalyzed by this enzyme was shown to have a specific requirement for cytidine nucleotide and the product was identified as cytidine monophosphate 2-aminoethylphosphonic acid (CMP-AEP).
3. The presence of CMP-AEP in Tetrahymena has been demonstrated and the concentration of this compound in the cells was determined.
4. A marked difference between the cytidyltransferases from rat liver and Tetrahymena has been observed; they were shown to operate under different pH optima and to have different affinity towards AEP as a substrate.
5. The synthesis in vitro of an "AEP-cephalin" from CMP-AEP and dipalmitin by a Tetrahymena cell-free preparation has been demonstrated.



### 1. Introduction

It has been established that the enzymic synthesis of lecithin and phosphatidylethanolamine is mediated by cytidine coenzymes (Kennedy and Weiss, 1956). The following reactions were shown to be catalyzed by enzymes prepared from guinea pig liver:



The enzyme which catalyzes reaction (1) was named PE-cytidyltransferase whereas the enzyme responsible for reaction (2) was named PE-diglyceride transferase. These two enzymes were subsequently found to be widely distributed in nature; their presence was shown in yeast and in various tissues of the rat, hog, guinea pig and other species.

Bremer, Figard and Greenberg (1960) have observed that, when  $[3\text{-}^{14}\text{C}]$ serine was injected into rats, the short-term incorporation of radioactivity into phospholipid ethanolamine was much higher than into PE or CDPE. This suggested a pathway for phosphatidylethanolamine formation which did not involve PE or CDPE as intermediates. Wilson, Gibson and Udenfriend (1960) incubated labelled serine with liver preparations and showed that the label acquired by phosphatidylethanolamine was higher than that in ethanolamine or in PE and that the degree of labelling was not significantly reduced by the addition of unlabelled ethanolamine or PE.

This pathway was eventually shown by Borkenhagen, Kennedy and Fielding (1961) to involve an exchange of

serine with the ethanolamine moiety of phosphatidylethanolamine, and is now the basis of the well-known serine decarboxylase cycle. This sequence does not contribute in the de novo synthesis of cepahlin, which is the function of the reaction sequence (1) and (2) shown above.

In support of this scheme, CDPE was isolated from the liver of the rat and of the hen, and the concentrations in these tissues have been determined by the isotope dilution technique (Kennedy and Weiss, 1956). CDPE has also been found in yeast (Kennedy and Weiss, 1956), and in cat brain (Kennedy, 1957). The wide distribution of CDPE in nature strongly supports the view that this nucleotide is an intermediate in cephalin biosynthesis in vivo.

Some of the work dealt with the specificity of the cytidyltransferases. Thus, Borkenhagen and Kennedy (1957) reported the presence of at least two cytidyltransferases in animal tissues. They showed that the activity of a partially purified enzyme preparation towards PE was lost on heating the enzyme briefly at 55°, while the activity towards phosphorylcholine remained unaffected. In a subsequent publication, Kennedy, Borkenhagen and Smith (1959) showed that deoxycytidine triphosphate was as effective as CTP in the presence of a preparation of phosphorylcholine cytidyltransferase. The deoxynucleotide was, however, less effective towards PE in the presence of a preparation of PE-cytidyltransferase.

Ansell, Chojnacki and Metcalfe (1965) reported that, in rat liver, the cytidyltransferase activity towards phosphorylpropanolamine was about one-twentieth of that exhibited with phosphorylethanolamine. No activity towards the former compound was found in rat brain homogenate.

On the other hand, the enzymes transferring the phos-



phorylated amino alcohol moiety from their cytidine diphosphate derivatives to diglyceride were reported to display low substrate specificity (Chojnacki and Korzybski, 1963; Chojnacki, 1964; Ansell et al., 1965). In many instances, the amount of "unnatural" phosphorylated base transferred from its cytidine diphosphate derivative to diglyceride was as great as with the derivatives of the naturally occurring choline and ethanolamine (Chojnacki, 1964).

The structural similarity of AEP to PE (cf. formulae I and II, Chapter I) and therefore of AEP-cephalin to phosphatidylethanolamine (cf. formulae V and VI, Chapter II), added to the knowledge of the occurrence of an AEP-cephalin in Tetrahymena, prompted the present search for a CTP-mediated pathway for AEP incorporation into a phosphonic analogue of phosphatidylethanolamine.

The incorporation of AEP into the lipids of Tetrahymena was investigated both in vivo and in vitro, and it was demonstrated that AEP-cephalin could be synthesized from AEP, with a phosphonic analogue of CDPE (which was designated CMP-AEP) as an intermediate. The occurrence of CMP-AEP in Tetrahymena has also been demonstrated.

Because of the lack of specificity displayed by some of the enzymes responsible for the synthesis of phospholipids, it was of importance to establish that the synthesis of AEP-containing phosphatides in Tetrahymena was a specific activity directed towards AEP, rather than a result of the activity of low-specificity enzymes, primary involved in the synthesis of phosphatidylethanolamine. With this aim in mind, some kinetic parameters, such as the apparent  $K_m$  for AEP and PE, and the respective maximal velocities with the two substrates, were determined for the cytidyltransferase preparation from Tetrahymena.

For comparison purposes, some properties of the

cytidyltransferase of rat liver were also examined.

## 2. Materials and Methods

### A. Materials

CMP-AEP (the phosphonic analogue of CDPE) was synthesized by the condensation of CMP and AEP in the presence of N,N'-dicyclohexylcarbodiimide (DCC) according to the method described by Kennedy (1956). The yield, after purification by chromatography on Dowex-1 (formate) was 16%, based on AEP.

Microsomal CDPE-pyrophosphatase (Allen and Rosenberg, personal communication) from chicken gut mucosa was a gift from Mr. A.K. Allen.

[<sup>14</sup>C-Ethanolamine]CDPE was prepared and supplied by Mr. A.K. Allen in our laboratory.

A crude cytidyltransferase was prepared from rat liver as follows: A rat weighing 250 g was killed by decapitation and bled. The liver was removed, weighed and homogenized at 0° with 4 volumes of 0.05 M Tris-HCl (pH 7.4) in a Potter-Elvehjem-type teflon homogenizer. The homogenate was centrifuged at 105,000 g for 1.5 h. and the supernatant was collected. The supernatant was brought to 0.5 saturation with ammonium sulphate and centrifuged at 500 g for 10 min. The precipitate was dissolved in a small volume of 0.45 M NaCl and dialyzed against 0.01 M Tris-HCl (pH 7.4) at 3° overnight. This preparation, which contained 10 mg protein/ml, was diluted with 0.01 M Tris-HCl to a desired concentration before use.

[<sup>32</sup>P]PE was a gift from Mr. A.K. Allen in this laboratory and was prepared from [<sup>32</sup>P]orthophosphate and ethyleneimine according to the method described by Christensen (1940) with some modifications (Hagerman, Rosenberg, Ennor, Schiff and Inoue, 1965).



The preparation of [ $^{32}$ P]AEP was described in Chapter II.

#### B. Methods

Cell-free homogenates and various cell fractions of Tetrahymena were prepared as follows: Log phase cells were harvested by low-speed centrifugation (500 g, 5 min.) and washed once with 0.9% (w/v) NaCl solution. The cells were disrupted by successive freezing and thawing of the suspension (one volume of packed cells in four volumes of 0.05 M Tris-HCl buffer, pH 7.4 containing 0.2 M sucrose), followed by homogenization in a Potter-Elvehjem-type glass homogenizer fitted with a teflon pestle. The homogenate was fractionated by differential centrifugation. Cell debris and nuclei were sedimented and removed after centrifuging for 10 min. at 500 g. The fraction that sedimented after 15 min. at 12,000 g was defined as mitochondrial, while that sedimented after 90 min. at 105,000 g was taken as microsomal.

The assay of [ $^{32}$ P]CMP-AEP produced in reaction mixtures was carried out as described by Borkenhagen and Kennedy (1957) with slight modifications. The reaction mixture (1 ml) contained (in  $\mu$ moles) Tris-HCl (pH 7.4), 50; [ $^{32}$ P]AEP, 3.5; CTP, 1.0;  $MgCl_2$ , 10; cysteine, 10; and the enzyme preparations. The mixture was incubated at 35°, the reaction was stopped by the addition of 5 ml of 5% (w/v) trichloroacetic acid and the precipitate was removed by centrifugation. To the protein-free supernatant was added 2 ml of a suspension of Norit A charcoal in water (20 mg/ml). After thorough mixing, the suspension was left in ice bath for 1 hour and the charcoal was removed by centrifugation. The charcoal was washed four times with 5 ml portions of water containing 1 mM serine. It was finally suspended in 5 ml of 1% (w/v) casein solu-

tion. An aliquot of the suspension was pipetted into a planchette, dried and counted in the end-window counter.

Measurement of the apparent  $K_m$  for AEP or PE and of  $V_{max}$  (Morrison, 1965) of cytidyltransferase of Tetrahymena and rat liver preparations was carried out with the concentration of CTP fixed at 1 mM. The enzyme activities were measured as follows: The incubation mixture (1 ml) contains  $MgCl_2$  (10 mM), K-phosphate buffer (pH 6.6) (100 mM), CTP (1 mM), enzyme protein (0.1 mg) and varying concentrations of [ $^{32}P$ ]AEP or [ $^{32}P$ ]PE. The tubes were incubated (at 37° with the rat preparation and 35° with the Tetrahymena preparation) for 10 min. and the reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The formation of [ $^{32}P$ ]CMP-AEP or [ $^{32}P$ ]CDPE in the reaction mixture was measured by the charcoal absorption method described above. Each experiment was run for two time periods to ensure that initial velocities were being measured.

In order to measure radioactivity incorporated into the trichloroacetic acid-insoluble material, the precipitate from the reaction mixture was suspended in a known volume of chloroform-methanol (1:1, v/v); a sample of the suspension was transferred to a planchette, dried and counted in the usual manner.

Dipalmitin (mixed isomers of 1,2- and 1,3-di-O-palmitylglycerol), when used as a substrate, was always added in an emulsified form. The emulsions were prepared as follows: 50 mg of Tween 20 and 60 mg of dipalmitin were weighed into a tube, followed by a drop of ethanol and few drops of water. The mixture was sonicated until all the particles disappeared and a transparent emulsion was obtained. This was then diluted to the desired volume with water.

Silicic acid-impregnated paper for the chromatography

of lipids was prepared according to the method described by Rouser, Bauman, Nicolaides and Heller (1961).

Paper chromatography on Whatman 3 MM paper was carried out using the following solvent systems:

- I. Water-saturated phenol, acetic acid, ethanol (100:10:12, by vol.).
- II. n-Butanol, acetic acid, water (4:1:5, by vol.), upper layer used.
- III. 0.02 M Acetic acid in 60% (v/v) aqueous ethanol.
- IV. 0.025 M Tris-HCl buffer (pH 8.0) in 50% (v/v) aqueous ethanol.
- V. Ethanol, formic acid, water (9:1:2, by vol.).
- VI. Diisobutylketone, acetic acid, water (40:20:3, by vol.).  
(For silicate coated papers).

Protein was estimated by the biuret reaction (Gornall, Bardawill and David, 1949).

### 3. Results

#### A. Incorporation of 2-aminoethylphosphonate (AEP) into phosphatide by suspensions of Tetrahymena

Uptake experiments showed that Tetrahymena cells displayed a high degree of selectivity towards compounds present in the medium. AEP uptake (followed with [ $^{32}$ P]AEP) under normal condition was slow. However, if the phosphorus content in the medium was reduced, and the cell suspension was shaken for 5 hours, uptake of AEP did occur.

Log phase cells grown in 300 ml of the complex medium were collected and the pellet (2.5 g of wet packed cells) was resuspended in 30 ml of the "defined" medium containing  $10^{-4}$  M phosphate and [ $^{32}$ P]AEP (3.5  $\mu$ moles,  $10^6$  cpm). The suspension was shaken at 35° for 5 hours. The degree of uptake of AEP from the medium was determined by measuring the radioactivity in the medium at the beginning and end



of the incubation. The results (Table III.1) indicate that 11% of the total radioactivity was present in the cells at the end of the incubation period. After incubation, 1.5 ml of 100% (w/v) trichloroacetic acid was added and the precipitate removed by centrifugation. The precipitate was washed three times with 5% (w/v) trichloroacetic acid, and the lipid was extracted as described elsewhere ("Methods", Chapter II). The lipid fraction was found to contain considerable radioactivity, which amounted to 76% of the [ $^{32}$ P]AEP taken up by the cells. Saponification of the lipid according to Dawson (1960) caused 80% of the radioactivity to become water soluble (Table III.1). These water-soluble products (saponifiable) were isolated and subjected to paper chromatography in solvent system I. Autoradiography of the chromatogram revealed a single radioactive band. The corresponding area on the paper was cut out and eluted with water. After purification by passage through Dowex-1 (acetate) and Dowex-50 ( $H^+$ ) columns and elution with water, the material was again chromatographed in solvent system I and II, with authentic glyceryl-AEP as a reference compound. The chromatograms were treated with ninhydrin and exposed to X-ray film. Only one spot was detected on each chromatogram and this gave an  $R_F$  of 0.63 and 0.15 in solvents I and II, respectively, in each case corresponding to that of glyceryl-AEP. The ninhydrin-staining areas in all cases corresponded with radioactivity.

B. Detection of nucleotide-bound AEP in Tetrahymena.

i. Presence of charcoal-absorbable AEP-derivative in Tetrahymena.

Log phase cells from a 600 ml culture in the "complex" medium were collected and the pellet (5 g of wet packed cells) was resuspended in 100 ml of the defined medium containing  $3 \times 10^{-4}$  M phosphate labelled with 20  $\mu$ C of



Table III.1. Distribution of radioactivity in Tetrahymena after incubation of the cells in a medium containing  $[^{32}\text{P}]\text{AEP}$

		Radioactivity (cpm)	%
Medium	Before incubation	$10^6$	100
	After incubation	$8.9 \times 10^5$	89
Cells	Total	$1.1 \times 10^5$	11
	Lipid Fraction	83,500	8.3
	Saponifiable Lipid	66,800	6.6
	Non-saponifiable Lipid	16,700	1.6

[<sup>32</sup>P]orthophosphate. The suspension was shaken at 35° for 1 hour, the cells were collected by centrifugation and washed once with 40 mM NaCl. The pellet was extracted with 50 ml of 5% (w/v) trichloroacetic acid and the extract shaken with 1.5 g of acid-washed charcoal (Norit A) for 30 min. The charcoal was then filtered off and washed four times with 5% (w/v) trichloroacetic acid containing 1 mM serine and 1 mM orthophosphate. After filtration, the charcoal was suspended in 30 ml of 6N HCl and the suspension was refluxed at 110° for 48 h. The charcoal was filtered off and the filtrate was taken to dryness. The residue was dissolved in water and applied to a column (3 cm x 10 cm) of Dowex-1 (acetate). The column was washed with 200 ml of water and the effluent was discarded. The column was then eluted with 200 ml of 5% (v/v) acetic acid. The eluate, which contained radioactive material, was taken to dryness and the residue was dissolved in water and again applied to a column (1.5 cm x 17 cm) of Dowex-1 (acetate). After washing with water, the column was eluted by the application of an increasing concentration gradient of acetic acid, using 1% (v/v) acetic acid in the main chamber and 350 ml of water in the mixing chamber. The eluate was collected in 10 ml fractions and the radioactivity of the effluent was assayed. Two radioactive peaks (Fig. III.1) were found to emerge on elution. The fractions comprising each peak were pooled and the solutions were taken to dryness. The residues were dissolved in water and subjected to paper electrophoresis at pH 2.0 (cf. "Methods", Chapter II), followed by autoradiography. The result showed that the second peak (peak B, Fig. III.1) contained a radioactive compound which was ninhydrin-positive and migrated as AEP. The first peak (peak A, Fig. III.1) contained material which

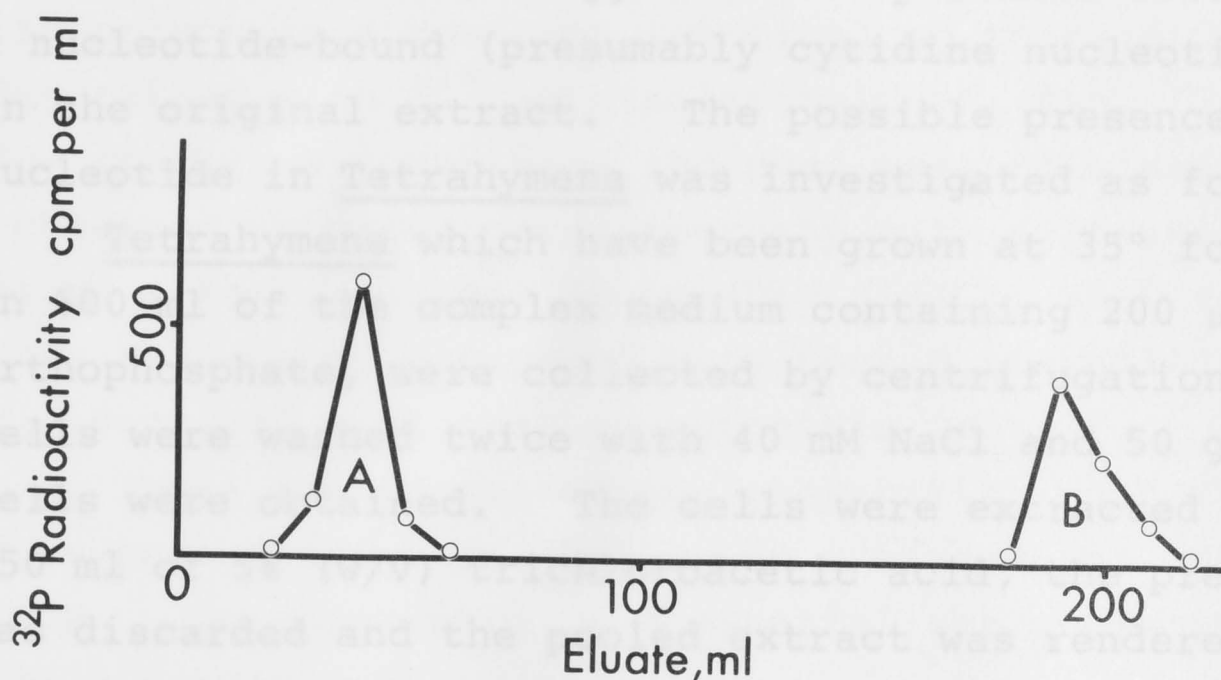


Fig. III.1. Chromatography on Dowex-1 of the hydrolysate of acid-soluble, charcoal-adsorbable material from Tetrahymena cells which had taken up [ $^{32}$ P]orthophosphate. The acid-free hydrolysate was applied to a column (1.5 cm x 17 cm) of Dowex-1 (acetate) and eluted with an increasing concentration gradient of acetic acid.



was ninhydrin-negative and remained on the origin during electrophoresis. It was not further investigated.

ii. The isolation and identification of CMP-AEP from Tetrahymena extracts.

Since exploratory experiments showed that free AEP was not adsorbed on charcoal under the conditions described above, these results suggested the possible occurrence of a nucleotide-bound (presumably cytidine nucleotide) AEP in the original extract. The possible presence of such a nucleotide in Tetrahymena was investigated as follows.

Tetrahymena which have been grown at 35° for 40 hours in 600 ml of the complex medium containing 200  $\mu$ C of [ $^{32}$ P]-orthophosphate, were collected by centrifugation. The cells were washed twice with 40 mM NaCl and 50 g wet packed cells were obtained. The cells were extracted twice with 150 ml of 5% (w/v) trichloroacetic acid, the precipitate was discarded and the pooled extract was rendered acid-free by three extractions with an equal volume of ether. The aqueous phase was mixed with an equal volume of ethanol and allowed to stand for 2 h. at 3°. The mixture was then centrifuged and the precipitate (mostly glycogen) discarded. The supernatant was taken to near dryness and diluted with 50 ml of water. The solution was passed through a column (2 cm x 20 cm) of Amberlite-IRC-50 ( $H^+$ ) followed by 120 ml of water. The eluate was taken to dryness in a Büchi rotatory evaporator, the water bath temperature being kept under 40°. The residue was dissolved in 10 ml of water and the solution was applied to a column (2 cm x 20 cm) of Dowex-1 (acetate) and washed with 120 ml of water. The column was then eluted with 120 ml of 5% (v/v) acetic acid and the eluate was taken to dryness. The residue was dissolved in 10 ml of water, and re-applied to a column of Dowex-1 (formate). The adsorbed material



was then eluted by the application of an increasing concentration gradient of formic acid. Formic acid eluate from the column (Fig. III.2, a) contained two major ultraviolet-light-absorbing peaks. The absorbancy ratio of 280 m $\mu$ /260 m $\mu$  of peak A (120-150 ml) was 1.33 and of peak B (190-250 ml) 2.0, which suggested that the latter might be a cytidine derivative. Furthermore, this peak emerged from the column in a position identical to that of CDPE, as determined with [ $^{14}$ C-ethanolamine]CDPE (Fig. III.2, b). The fractions comprising the peak were combined and subjected to paper chromatography with authentic CMP-AEP as a marker. It was found (Fig. III.3) that in two solvent systems the compound from peak B travelled with an  $R_F$  identical with that of authentic CMP-AEP.

There was thus a strong possibility that the compound from peak B (Fig. III.2, a) was a mixture of CMP-AEP and CDPE. To check this, the material was subjected to controlled hydrolysis and products were examined. A portion of the material was subjected to "drastic" acid hydrolysis, and the hydrolysate, after being made acid-free, was examined by paper electrophoresis and autoradiography. Only two radioactive compounds were detected, and they behaved as AEP and orthophosphate, respectively.

Another portion of the material was dissolved in 1 ml of 0.05 M Tris-HCl (pH 8.0) containing 1 mM EDTA. To the mixture was added 0.2 ml (2 mg protein) of a microsomal CDPE-pyrophosphatase preparation from the chicken-gut (cf. "Materials") and the mixture was incubated at 37° for 90 min. The incubation was stopped by the addition of 0.05 ml of 100% (w/v) trichloroacetic acid. After centrifugation, the precipitate was discarded and the supernatant was rendered acid-free by extraction with ether. The aqueous layer was separated and taken to dryness. This

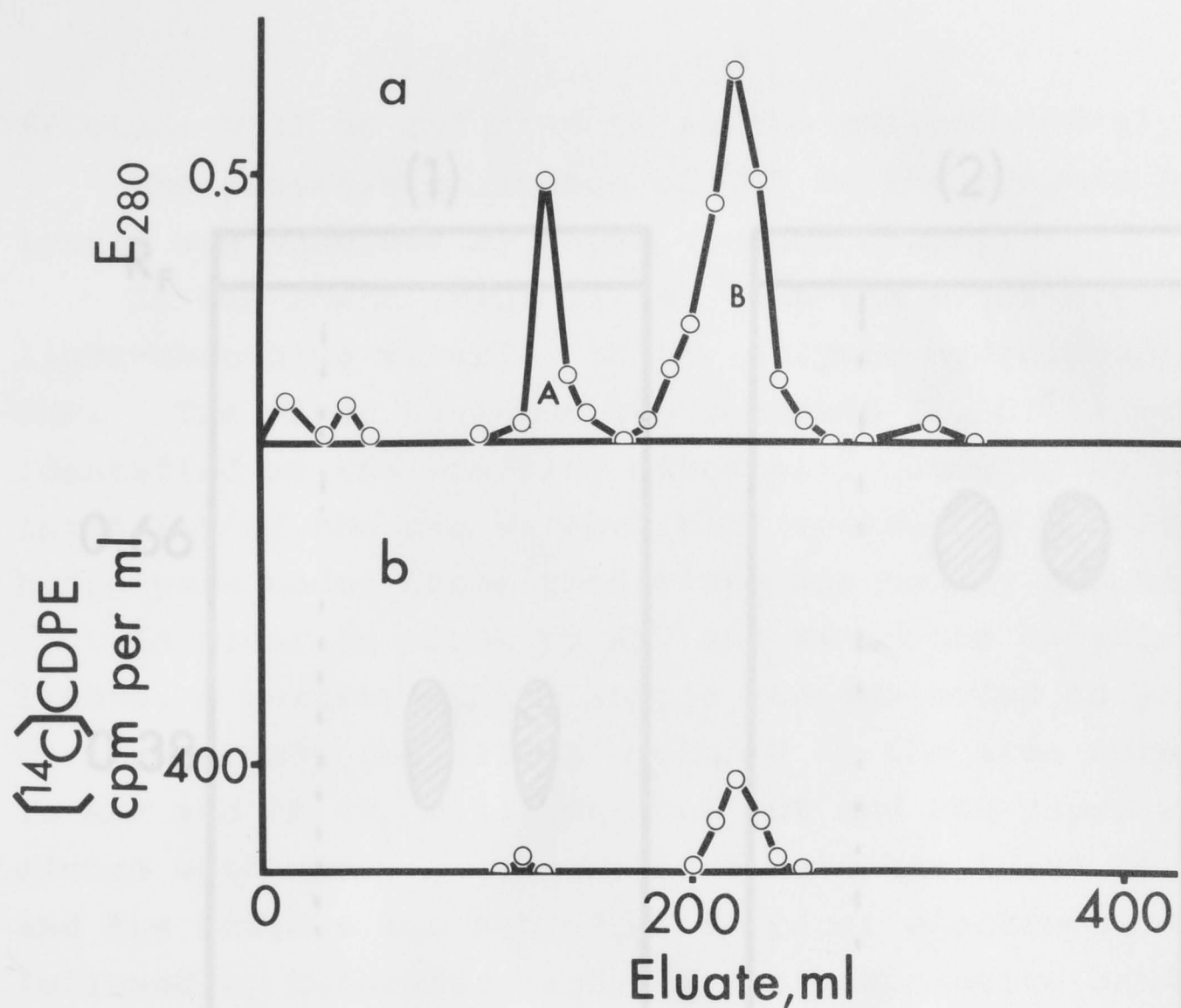
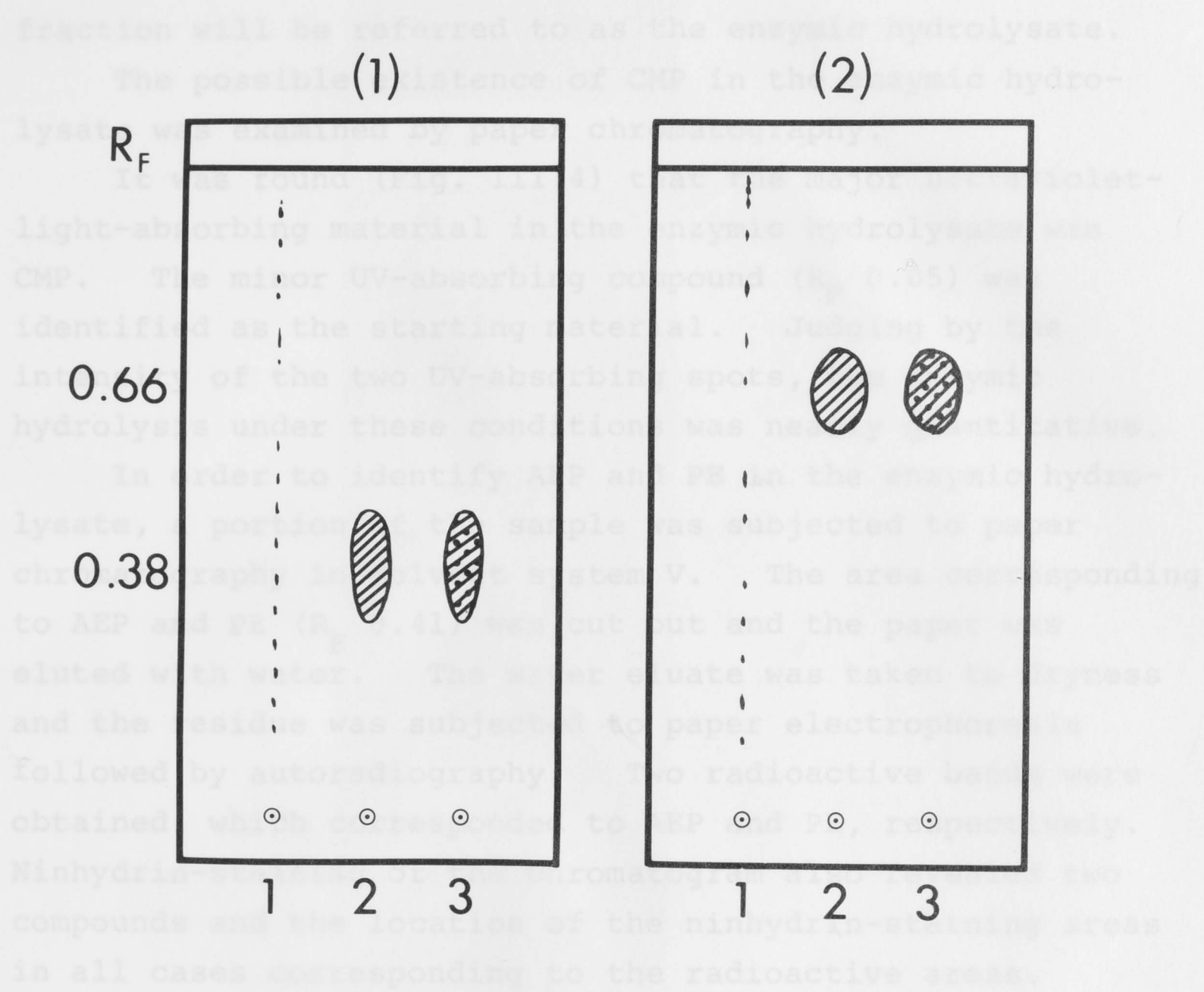


Fig. III.2. Emergence of ultraviolet light-absorbing material (a) or radioactivity (b) on fractionation of the acid-soluble material from Tetrahymena.

To each of two columns (1.2 cm x 17 cm) of Dowex-1 (formate) was applied a solution of partially purified Tetrahymena extract (see text) alone (a) or supplemented with [<sup>14</sup>C-ethanolamine]-CDPE (b). The columns were eluted by the application of an increasing concentration gradient of formic acid (0.04 N formic acid in the supply vessel and 300 ml of water in the mixing vessel). The effluent was collected in 10-ml fractions and the absorbance at 280 mμ (a) or radioactivity (b) were measured.



**Fig. III.3.** Paper chromatography of the ultraviolet-light-absorbing compounds from the two major peaks shown in Fig. III.2.

1. Compound from peak A. 2. Synthetic CMP-AEP. 3. Compound from peak B.

(1) Solvent system III. (2) Solvent system IV.

Hatched areas denote ultraviolet-light absorbing material. Spotted areas denote darkening of X-ray film after exposure to the chromatogram.

fraction will be referred to as the enzymic hydrolysate.

The possible existence of CMP in the enzymic hydrolysate was examined by paper chromatography.

It was found (Fig. III.4) that the major ultraviolet-light-absorbing material in the enzymic hydrolysate was CMP. The minor UV-absorbing compound ( $R_F$  0.05) was identified as the starting material. Judging by the intensity of the two UV-absorbing spots, the enzymic hydrolysis under these conditions was nearly quantitative.

In order to identify AEP and PE in the enzymic hydrolysate, a portion of the sample was subjected to paper chromatography in solvent system V. The area corresponding to AEP and PE ( $R_F$  0.41) was cut out and the paper was eluted with water. The water eluate was taken to dryness and the residue was subjected to paper electrophoresis followed by autoradiography. Two radioactive bands were obtained, which corresponded to AEP and PE, respectively. Ninhydrin-staining of the chromatogram also revealed two compounds and the location of the ninhydrin-staining areas in all cases corresponding to the radioactive areas.

The nucleotide pyrophosphatase-catalyzed hydrolysis of the compounds from peak B (Fig. III.2, a) is represented by the following equations:



Quantitative analysis of AEP in the mixture showed that Tetrahymena cells (in the stationary phase) contained about 30  $\mu\text{moles}$  of CMP-AEP per gram of wet packed cells.



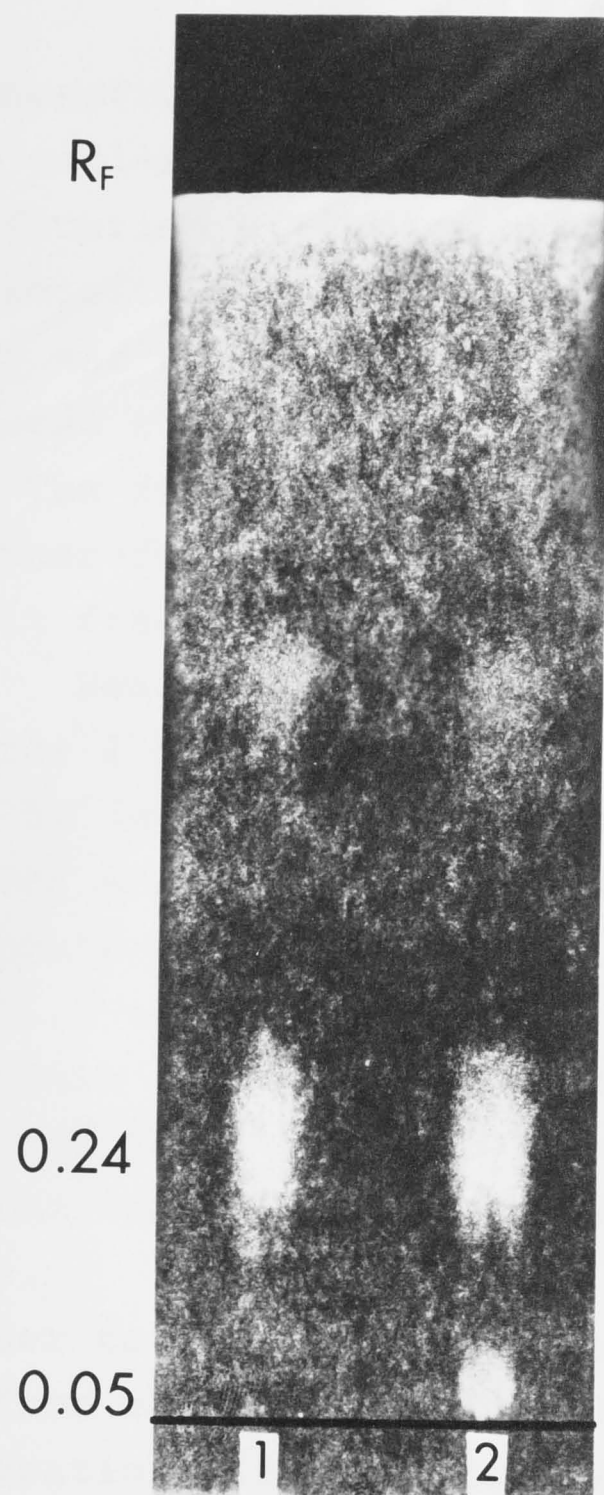


Fig. III.4. Paper chromatogram of the enzymic hydrolysate of the material from peak B (Fig. III.2a) (the paper was developed in the solvent system V).

1. Authentic CMP
2. Sample from the enzymic hydrolysate

The spots were detected by ultraviolet-light absorption.

C. Demonstration of AEP-cytidyltransferase activity in cell-free preparations of Tetrahymena and identification of the reaction product as CMP-AEP.

The demonstration of the occurrence of CMP-AEP in Tetrahymena and the structural similarity of CMP-AEP to CDPE suggested that it may be synthesized in a way similar to CDPE. The formation of CMP-AEP by cell-free preparations was therefore investigated. Homogenates and the various cell fractions were prepared as described in "Methods". Reaction mixtures (cf. Table III.2) were incubated for 1 h. at 35° and precipitated by the addition of 5 ml of 5% (w/v) trichloroacetic acid. The protein-free extracts were treated with charcoal as described before and the appearance of charcoal-held radioactivity was assayed (cf. "Methods").

The result (Table III.2) showed that only the complete homogenate and the high-speed supernatant fraction showed activity, and that the activity in the supernatant was heat-labile.

In order to remove completely any low-molecular weight cofactors from the preparation, the supernatant was brought to 0.5 saturation with ammonium sulphate, the precipitate was collected, dissolved in 0.45 M NaCl and dialyzed against 100 volumes of 0.01 M Tris-HCl (pH 7.4) overnight. Activities were assayed using the incubation mixture described above, except that Tris-HCl was replaced by 3,3-dimethylglutarate (pH 6.6) and the incubation time was reduced to 10 min.

It was found (Table III.3) that the single-step precipitation with ammonium sulphate increased the total enzyme activity to 170% and its specific activity to 6 times that of the original supernatant fraction.

The precipitate from the 0.5-saturation with ammonium

Table III.2. Conversion of [ $^{32}$ P]AEP to a charcoal-adsorbable compound by cell fractions of Tetrahymena

Reaction mixtures (1.0 ml) contained (in  $\mu$ moles) Tris-HCl (pH 7.4), 50;  $MgCl_2$ , 10; cysteine, 10; CTP, 1.0; AEP, 3.5 ( $5 \times 10^5$  counts/min) and the enzyme preparation. Controls received trichloroacetic acid at zero time. Incubation, 1 h. at 35°.

Fraction	Activity : $\mu$ moles of AEP converted to a charcoal-adsorbable compound per 1.0 g wet packed cells
Homogenate	164
Mitochondria	0
Microsomes	3.4
Supernatant	1360
Supernatant control	0
Mitochondria + boiled supernatant	0
Microsomes + boiled supernatant	7.0

Table III.3. Purification of the AEP-cytidyltransferase from Tetrahymena by ammonium sulphate precipitation (10 g wet-packed cells were used).

Fraction	Volume (ml)	Concentration Units*/ml	Total Units*	Protein mg/ml	Specific Activity Units/mg	Yield %
Crude Supernatant	35	1.76	61.5	6.92	0.254	100
Precipitate from 0.5-saturation with $(\text{NH}_4)_2\text{SO}_4$	10	10.35	103.5	7.12	1.452	170

\*1 unit = 1  $\mu$ mole CMP-AEP formed per h.



sulphate was retained and was used in all subsequent work.

Specificity for nucleotide. The enzyme was found to be strictly specific for cytidine nucleotide, CTP being the only nucleotide used (Table III.4).

The remaining nucleotide triphosphates, when present at the same concentration, were not utilized.

Identification of the reaction product as CMP-AEP.

A large-scale reaction mixture (see legend to Fig. III.5) was incubated for 1 hour and the reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5% (w/v). The precipitated material was removed by centrifugation and the supernatant was extracted four times with an equal volume of ether. The acid-free supernatant was fractionated by chromatography on Dowex-1 (Fig. III.5).

Two radioactive peaks emerged, the second of which coincided also with a peak of ultraviolet-light absorption. This peak was absent from a similarly treated control reaction mixture, where the trichloroacetic acid was added before the enzyme preparation. In the latter case only the first radioactive peak appeared. The fractions comprising the radioactive peaks (Fig. III.5) were pooled and the solutions were concentrated. Samples were chromatographed on paper in solvent system III and IV using CDPE and synthetic CMP-AEP (cf. "Materials") as references. The dried chromatograms were inspected for ultraviolet-light-absorbing areas. In each of the solvent systems (Fig. III.6) the incubation product (peak 2) showed the same  $R_F$  as the synthetic CMP-AEP. Autoradiography of the dried chromatograms showed the radioactive areas corresponding to the ultraviolet-light-absorbing areas of the sample. The material in the first peak behaved as AEP.

Table III.4. The specificity of the AEP-cytidyltransferase from Tetrahymena with respect to the nucleotide.

Reaction mixtures were as described in Table III.2, but each contained 4 mg of the dialyzed protein fraction precipitated from the high-speed Tetrahymena supernatant by 0.5 saturation with ammonium sulphate.

Nucleoside triphosphate used (1.0 mM)	Charcoal-bound radioactivity (counts/min)
ATP	140
CTP	28,800
GTP	0
ITP	0
UTP	90

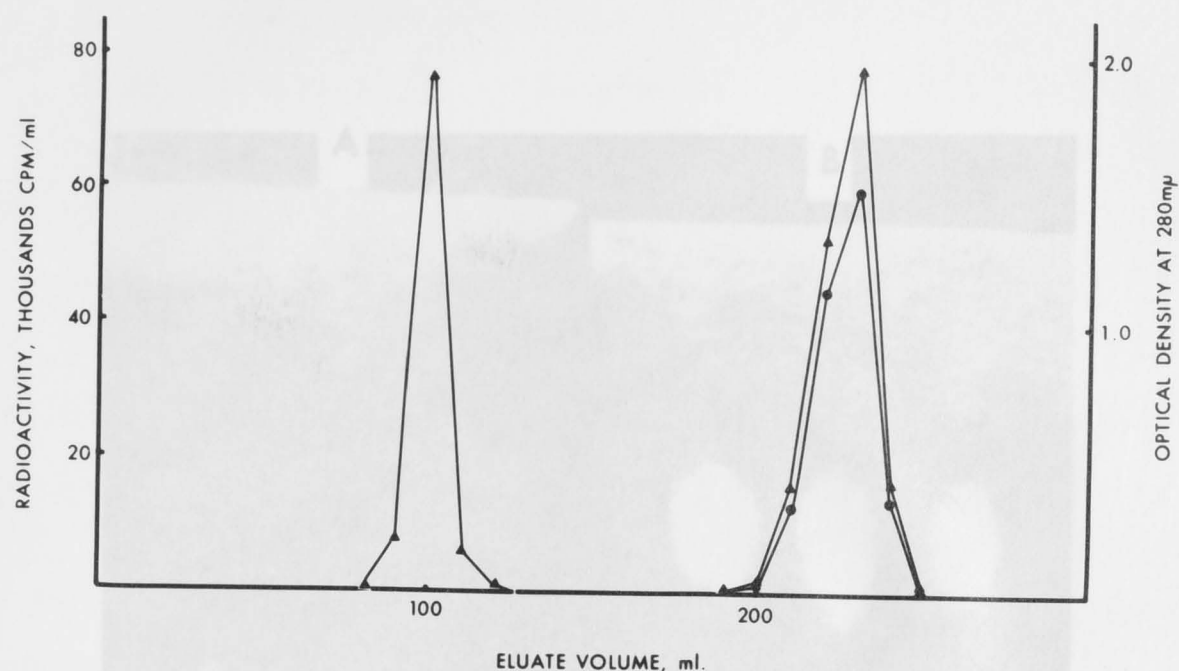


Fig. III.5. Emergence of radioactivity ( $\blacktriangle$ ) and ultraviolet-light-absorbing material ( $\bullet$ ) from Dowex-1 (formate) on fractionation of the protein-free material from the reaction of AEP and CTP. The reaction mixture (10 ml) contained (in  $\mu$ moles): Tris-HCl buffer (pH 7.4) 250;  $MgCl_2$ , 100; CTP, 10.0; cystine, 100;  $[^{32}P]AEP$ , 6.0 (specific radioactivity,  $5 \times 10^5$  counts/min per  $\mu$ mole) and 20 mg of the dialysed protein fraction precipitated from the high-speed *Tetrahymena* supernatant by 0.5 saturation with ammonium sulphate. The acid-free extract was applied to a column (1.5 cm x 17 cm) of Dowex-1x2 (formate), 200-400 mesh, and followed through with 100 ml of water. The column was then eluted with an increasing concentration gradient of formic acid, using 0.04 M formic acid in the main chamber and 300 ml of water in the mixing chamber. The entire procedure was carried out at 3°. The effluent was collected in 10 ml fractions, which were assayed for radioactivity and absorbance at 280  $m\mu$ .

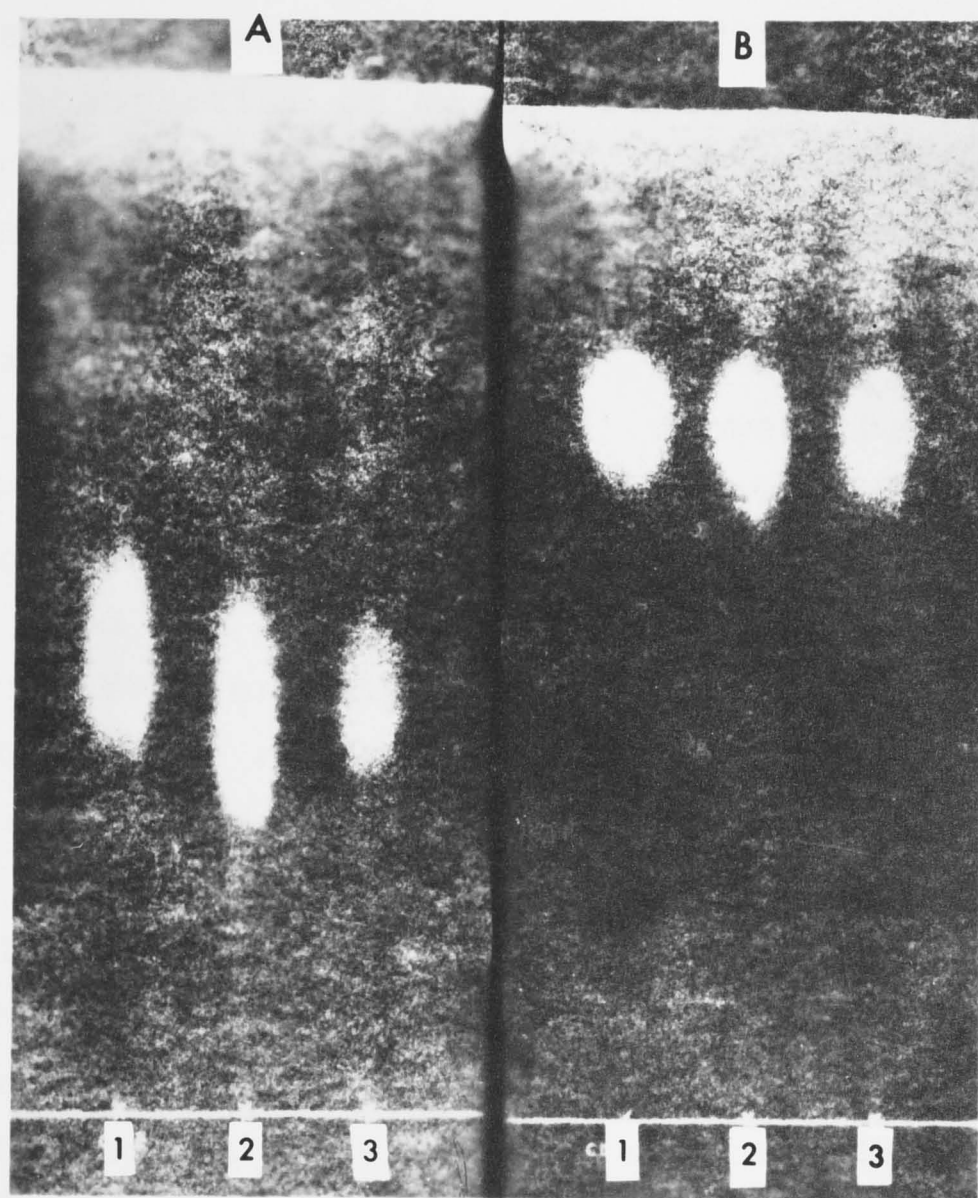


Fig. III.6. Chromatography of the ultraviolet-light-absorbing compounds from the second peak (Fig. III.5). (1) CDP-ethanolamine; (2) synthetic CMP-AEP; (3) product of enzymic reaction (second peak, Fig. III.5).

Solvent systems : A, III; B, IV.



#### D. Comparison of AEP- and PE-cytidyltransferase activities in Tetrahymena.

##### i. Kinetic parameters.

The apparent  $K_m$  values of AEP- and PE-cytidyltransferases for their respective substrates were measured with the concentration of Mg-CTP fixed at 1 mM and with varying concentrations of AEP or PE. The reciprocal plots of the initial velocities ( $1/v$ ) against substrate concentrations ( $1/S$ ) (Fig. III.7) were used for the calculation of the values of  $K_m$  and  $V_{max}$ .

The result (Table III.5) showed that the apparent  $K_m$  of AEP-cytidyltransferase for AEP was about five times higher than that of PE-cytidyltransferase for PE. On the other hand, in any given preparation from Tetrahymena, the  $V_{max}$  of AEP-transferase activity was higher than that of PE-transferase activity.

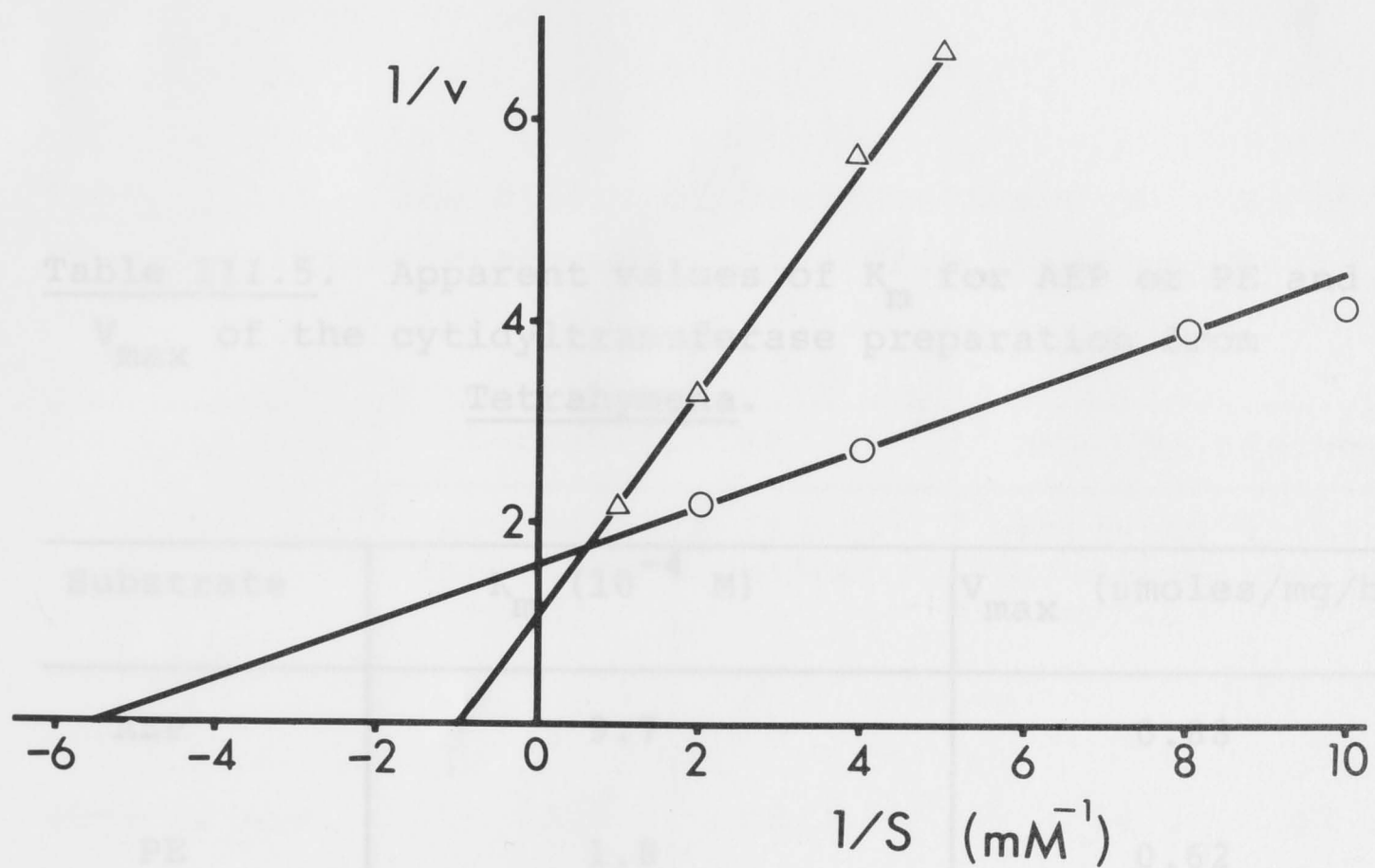
##### ii. Heat denaturation of the cytidyltransferase preparation from Tetrahymena.

In view of the structural similarity of AEP and PE, and the occurrence of both of these compounds and their CMP-derivatives (e.g. CMP-AEP and CDPE) in Tetrahymena, it appeared appropriate to test whether AEP- and PE-cytidyltransferase activities in Tetrahymena were due to one enzyme. One of the criteria tested was the lability of the two activities to heat denaturation.

It was found (Table III.6) that the two enzyme activities responded similarly to the heat treatment, both suffering considerable denaturation even under mild condition ( $45^\circ$ , 5 min.). It was thus not possible to differentiate between the two activities on the basis of this test.

#### E. Comparison of optimum pH for the activity of cytidyltransferases from Tetrahymena and rat liver.

Both AEP- and PE-cytidyltransferase activities of



**Fig. III.7.** Double-reciprocal plot of the initial velocity versus substrate concentration for the cytidyltransferases from Tetrahymena enzyme preparation. Initial velocity ( $v$ ) is expressed as  $\mu$ moles of CMP-AEP or CDPE formed per mg of enzyme protein per h.

$\Delta$  —————  $\Delta$  AEP transferase  
 $\circ$  —————  $\circ$  EP transferase

Table III.5. Apparent values of  $K_m$  for AEP or PE and  $V_{max}$  of the cytidyltransferase preparation from Tetrahymena.

Substrate	$K_m$ ( $10^{-4}$ M)	$V_{max}$ ( $\mu$ moles/mg/h.)
AEP	9.7	0.88
PE	1.8	0.62

*Tetrahymena* operates optimally at pH of 6.5 (Fig. III.5). The PE-cytidyltransferase activity of rat liver showed two pH optima, at pH 5.0 and pH 6.2, respectively, the enzyme activity at the former pH value being higher under the conditions of assay. No AEP-cytidyltransferase activity could be detected in the rat liver preparation in the assay system used. However, in a large scale incubation mixture (10 ml) with the  $[^{32}P]$ ATP-concentration

Table III.6. The effect of heat-treatment on the AEP- and PE-cytidyltransferase activities from *Tetrahymena*.

	PE-transferase		AEP-transferase	
	Activity (mmoles/mg/h.)	% Inactivation	Activity (mmoles/mg/h.)	% Inactivation
Control	525	0	630	0
40°, 10 min.	61.5	88.3	74.3	88.2
20 min.	53.0	89.9	88.0	86.0
45°, 5 min.	0	100	0	100

the table, the addition of dipalmitin from the reaction mixture resulted in a decrease in AEP incorporation into lipid of only about one-third. There appears thus to be a considerable amount of endogenous diglyceride in the preparation.

The products of the enzyme reaction were examined as follows. The lipid was extracted from a reaction mixture similar in composition to that described in Table III.7. The total radioactivity in the lipid fraction amounted to 2,500 counts per min. (1% of the CMP-AEP added). The



Tetrahymena operate optimally at pH of 6.6 (Fig. III.8). The PE-cytidyltransferase activity of rat liver showed two pH optima, at pH 8.0 and pH 6.2, respectively, the enzyme activity at the former pH value being higher under the conditions of assay. No AEP-cytidyltransferase activity could be detected in the rat liver preparation in the assay system used. However, in a large scale incubation mixture (10 ml) with the [ $^{32}$ P]AEP concentration increased to 2 mM, a slight amount of [ $^{32}$ P]CMP-AEP was formed, allowing the rate to be calculated as 1.6  $\mu$ moles per mg protein per hour. The value was less than one-hundredth of that of PE-cytidyltransferase activity in the same preparation.

F. The demonstration of CMP-AEP:diglyceride-AEP transferase activity in cell-free preparation of Tetrahymena.

In view of the demonstrated presence of an AEP-containing phosphatide, and the synthesis of CMP-AEP in Tetrahymena, attempts were made also to demonstrate synthesis of this type of phosphatide by following radioactivity incorporation from [ $^{32}$ P]CMP-AEP into lipid.

It was found (Table III.7) that this activity resides largely with the "mitochondrial" fraction. As seen from the table, the omission of dipalmitin from the reaction mixture resulted in a decrease in AEP incorporation into lipid of only about one-third. There appears thus to be a considerable amount of endogenous diglyceride in the preparation.

The products of the enzymic reaction were examined as follows. The lipid was extracted from a reaction mixture similar in composition to that described in Table III.7. The total radioactivity in the lipid fraction amounted to 2,500 counts per min. (1% of the CMP-AEP added). The

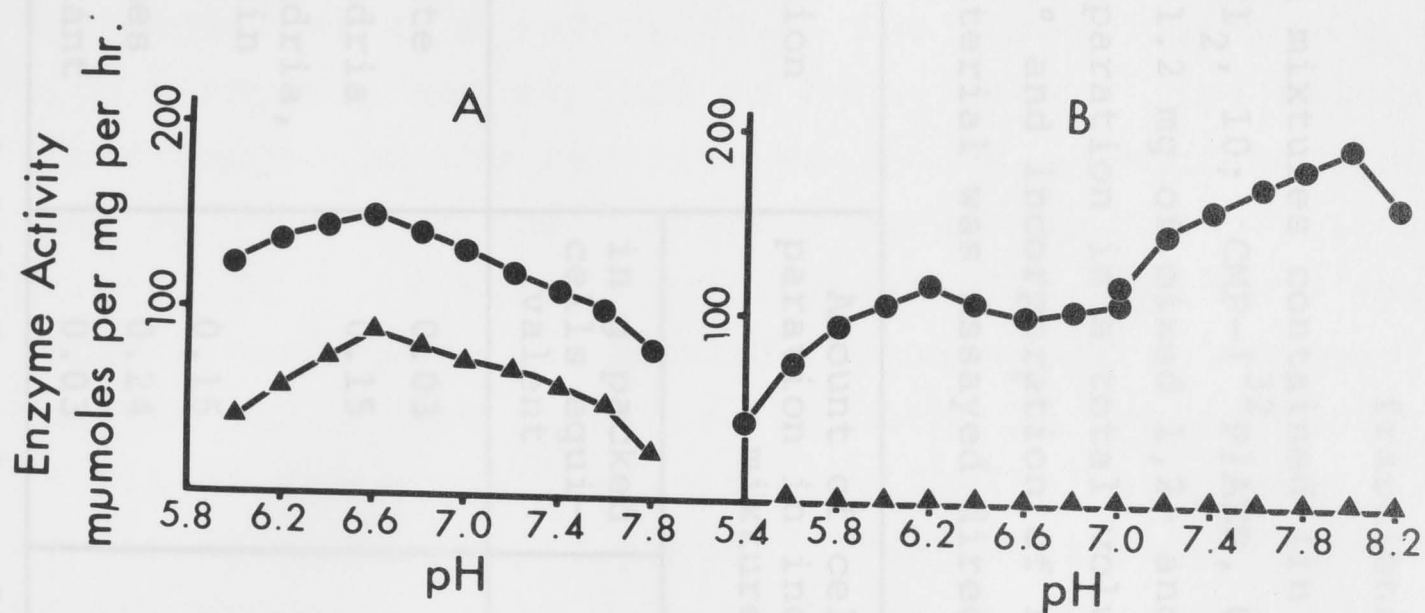


Fig. III.8. The effect of pH on the activities of cytidyl transferase preparations from *Tetrahymena* and rat liver.

A. *Tetrahymena* preparation

B. Rat liver preparation

Buffers used:

K-phosphate; pH 5.8-7.4

$\beta, \beta'$ -Dimethylglutaric acid; pH 5.4-7.0

NEM-HCl; pH 7.0-8.2

● ————— ● EP Transferase

▲ ————— ▲ AEP Transferase

Enzymic activities were measured as described in "Methods" except that various buffers were used (at 250 mM) and the substrate (AEP or EP) concentration was fixed at 1 mM.

Table III.7. Incorporation of radioactivity from CMP-AEP into lipid by cell fractions from Tetrahymena.

Reaction mixtures contained (in  $\mu$ moles) Tris-HCl buffer (pH 7.4), 50; cysteine, 10;  $\text{MgCl}_2$ , 10; CMP- $^{32}\text{P}$ AEP, 0.13, specific activity  $3.7 \times 10^5$  counts/min per  $\mu$ mole; 1.2 mg of mixed 1,2- and 1,3-dipalmitin (in emulsified form) and the cell preparation in a total volume of 1.0 ml. Mixtures were incubated for 1.5 h. at  $35^\circ$  and incorporation of radioactivity into trichloroacetic acid-precipitable material was assayed directly.

Fraction	Amount of cell pre- paration in incubation mixture		Counts/min in acid-insoluble material		$\mu$ moles CMP-AEP con- verted to trichloro- acetic acid-insoluble product per hour	
	in g packed cells equi- valent	in mg protein	Control*	Test	per g cells equivalent	per mg protein
Homogenate	0.03	4.5	1	161	9.6	0.063
Mitochondria	0.15	4.2	0	564	6.8	0.241
Mitochondria, dipalmitin omitted	0.15	4.2	0	413	4.9	0.176
Microsomes	0.24	8.4	0	117	0.9	0.025
Supernatant	0.03	2.4	0	0	0	0

\* Cell fraction added after trichloroacetic acid.



fat-free pellet contained no radioactivity above that of background. The lipid was chromatographed on silicic acid-impregnated paper in solvent system VI, with authentic dimyristoyl-glycerol-aminoethylphosphonic acid (synthetic AEP-cephalin) as a reference. The chromatogram was stained with ninhydrin and exposed to X-ray film. The radioactive lipid formed in the reaction was found to migrate with the same  $R_F$  (0.52) as the authentic reference compound, with a faint trail. A portion of the isolated lipid was saponified according to the procedure quoted (cf. "Methods", Chapter II). The procedure resulted in the solubilization of 81% of the total radioactivity, 19% of the counts remaining in the lipid phase. The water-soluble products of the saponification were isolated as described above and were subjected to paper chromatography in solvent systems II and III, followed by autoradiography of the dried, ninhydrin-stained paper. In all cases the saponified product of the reaction behaved as authentic glycerol-AEP which was used as a reference. The shape and location of the ninhydrin-positive spots on the papers corresponded exactly to the darkened areas of the autoradiographs (cf. Fig. III.9). "Mild" acid hydrolysis of this material yielded only one radioactive product which was identified as AEP.

#### 4. Discussion

Evidence has been presented for the existence of a pathway, although not a major one (cf. Chapter II), whereby the phosphonic analogue of phosphatidylethanolamine (AEP-cephalin) can be synthesized. This pathway does not differ from that described for the synthesis of phosphatidylcholine or phosphatidylethanolamine (Kennedy and Weiss, 1956), except that the phosphonate, rather than the ester



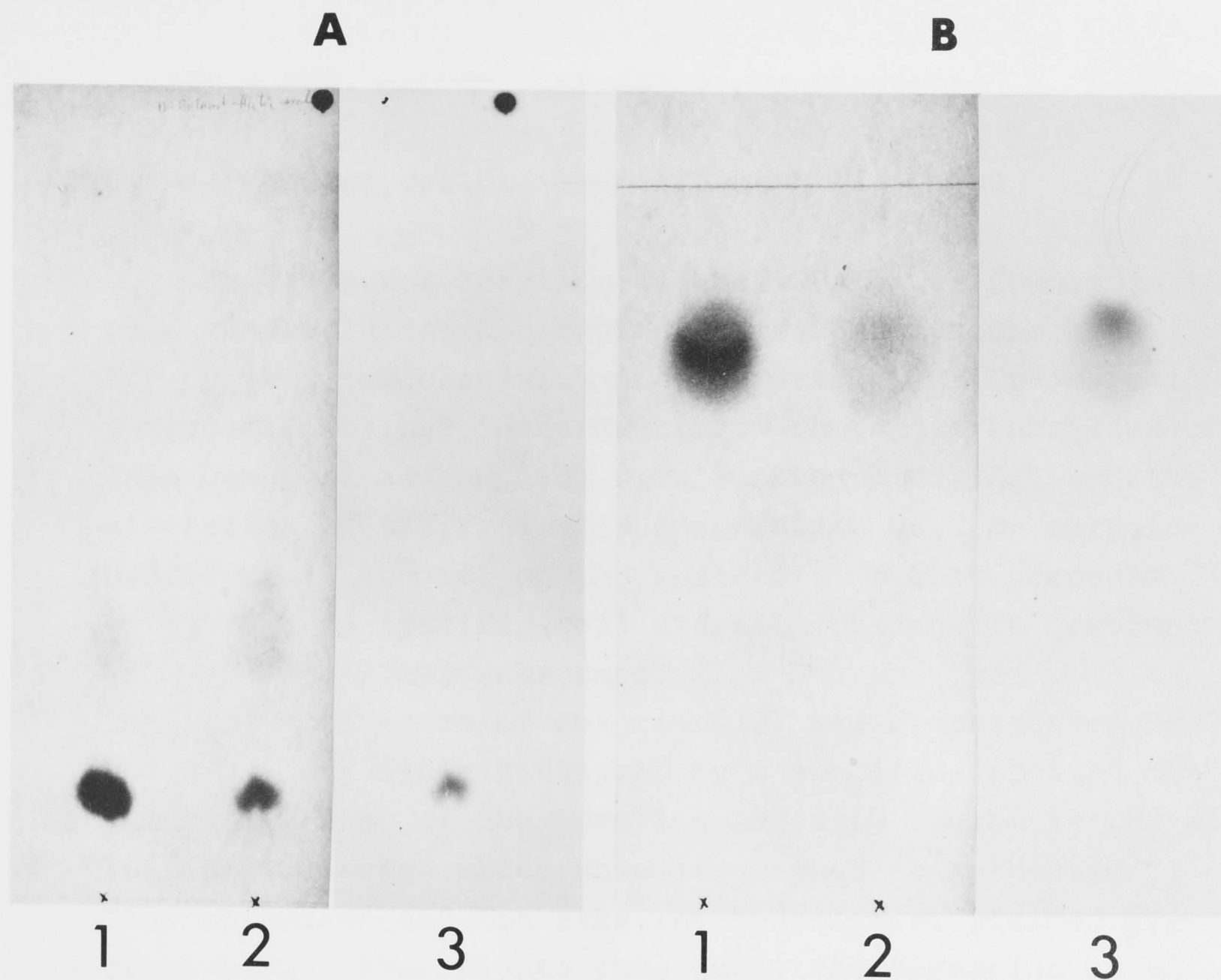


Fig. III.9. Paper chromatography of the saponification products of the lipid obtained in the enzymatic reaction between CMP-AEP and dipalmitin. (1) authentic glyceryl-AEP; (2) and (3) reaction product. Detection : (1) and (2) ninhydrin; (3) autoradiograph. Solvent systems : A, II; B, III.

phosphate of the base is utilized. The reactions involved can be represented as:



Evidence for Reaction (1) rests on: (a) the isolation of a charcoal-adsorbable product which contained both AEP-derived radioactivity and ultraviolet light-absorbing material; (b) the identical chromatographic behaviour of this compound as compared with authentic CMP-AEP and the liberation of AEP from this compound on acid or enzymic hydrolysis; and (c) on the synthesis of this compound in vitro by a specific cell fraction, using CTP (exclusively) and AEP as the substrates.

The occurrence of Reaction (2) was demonstrated by the transfer, again catalyzed by a specific fraction of the homogenate, of the labelled AEP from synthetic CMP-AEP to lipid material which chromatographed as authentic "AEP-cephalin" and, on alkaline hydrolysis, yielded glyceryl-AEP. The results thus imply the formation of an AEP-containing glycerophosphatide.

The kinetic work and other investigations into the nature of the AEP-cytidyltransferase activity of Tetrahymena did not yield sufficient data to allow comparison between this activity and the related PE-cytidyltransferase. Although the kinetic parameters such as  $K_m$  for AEP and EP and  $V_{max}$  show considerable differences, the results of the heat-treatment are inconclusive and the question whether the two activities are catalyzed by the same, or different enzymes, was not resolved. Since this aspect of the problem is only slightly relevant to the main theme of this

Thesis, it was not further investigated.

The apparent  $K_m$  values for the bases, AEP and PE, serve the purpose of comparison of the affinities of the respective sites for the substrates. These values do not represent real kinetic constants, since they were not derived under conditions of saturation with the other substrate, Mg-CTP, the  $K_m$  of which is not known.

It is of interest to note that under comparable conditions, the preparation displayed a higher affinity for PE, while the maximal velocity (at saturation) was considerably higher with AEP. The comparatively low activities with AEP in the pH-activity curves (Fig.III.8) are a result of assaying both activities at the same substrate concentration, rather than at saturating concentrations.

A marked difference between the cytidyltransferase from rat liver and that of Tetrahymena has been observed. The formation of both CMP-AEP and CDPE in preparations from Tetrahymena was maximal at pH 6.6, while the formation of CDPE in the rat liver enzyme preparation was maximal at pH 8.0; furthermore, only traces of CMP-AEP synthesis could be detected in this tissue.

Since AEP is a normal constituent in Tetrahymena, it is not surprising that there are enzymes in this organism which catalyze some turnover of AEP in the cells. In the rat, AEP is not a normal metabolite and the corresponding transferring system does not operate in this animal. A comparatively high concentration of AEP was necessary to show a small amount of CMP-AEP formation with the rat liver preparation. The amount of CMP-AEP thus formed was less than one-hundredth of the CDPE formed under the same conditions. The structural similarity of AEP and PE apparently allows the partial substitution of AEP in the



usual phospholipid-synthesizing system of the rat. The observation of the incorporation of AEP into rat liver lipid (Kandatsu et al., 1965) or into the lipid of the housefly (Bridges and Ricketts, 1966) can be similarly explained.

Another point deserving comment is the low degree of AEP-cytidyltransferase activity in the crude homogenate of Tetrahymena, compared to that in the supernatant fraction, and the further increase in activity after ammonium sulphate precipitation. A similar observation has been reported by Gurr, Brindly and Hübscher (1965), who showed that in the intestinal mucosa the activity of phosphorylcholine-cytidyltransferase in the crude homogenate was low. Only after fractionation of the particle-free supernatant with ammonium sulphate was there any clear evidence for the presence of this enzyme in the tissue. The only possible explanation for this result is the short life of the reaction product in the presence of all of the cell components. The utilization of CDP-choline for phospholipid synthesis and/or destruction by a particulate pyrophosphatase may well contribute to this phenomenon.

It should be noted that in the Reaction (2) catalyzed by the mitochondria (Table III.7), AEP incorporation from CMP-AEP into lipid was only partially decreased if diglyceride was omitted, so that absolute dependence on diglyceride could not be demonstrated. Unfortunately, all attempts to extract endogenous diglyceride from the enzyme preparation resulted in complete loss of activity.

One important aspect of the present results must not be overlooked. It has been shown previously (Rosenberg, 1964) and has been further confirmed by the more detailed investigation (cf. Chapter II), that free AEP in Tetrahymena cannot possibly be a precursor of the bulk of the



lipid-bound AEP, certainly not of the AEP-containing glycerophosphatide which, both in growing cultures and in washed cell suspension presented with [ $^{32}$ P]orthophosphate, attains in short periods a much higher specific radioactivity than does free AEP. The main pathway for the synthesis of the AEP-cephalin must therefore involve other reactions, and probably includes also the site of the biosynthesis of the C-P bond. The pathway described in the present work may serve merely as a salvage mechanism for the recovery of any free AEP that may arise as a result of the breakdown of some of the lipids or of other insoluble components. The origin of free AEP from such compounds is strongly indicated by the time course of labelling (Rosenberg, 1964). There is, furthermore, no evidence that the pathway described in the present work is at all specific for CMP-AEP. It is, indeed, very likely that CMP-AEP is simply used, as CDPE would be, by the usual phospholipid-synthesizing system which, in other species at least, has been shown to lack specificity with respect to the base (Chojnacki, 1964). Although AEP was not tested in those experiments, recent reports on the finding of AEP-containing lipid in ruminants which harbour protozoa (Shimizu et al., 1965; Kandatsu and Horiguchi, 1965) and the demonstration of the incorporation of administered AEP into the liver lipids of the rat (Kandatsu et al., 1965), seem to indicate that the mammalian systems cannot reject AEP as a source of phospholipid bases. Experiments have also shown that in a chicken-kidney microsomal preparation which synthesizes phospholipid and serine-ethanolamine phosphodiester (Rosenberg and Ennor, 1966), a phosphonate-containing diester analogue and phosphonolipid are synthesized if the system is presented with CMP-AEP, rather than CDPE, as a substrate (Allen and

Rosenberg, personal communication). Thus the transfer of AEP from CMP-AEP to phosphatide is not a reaction specific to Tetrahymena. On the other hand, potential for this reaction in this species is much higher than, for instance, in the rat, due to the specific ability to synthesize the required nucleotide reactant - CMP-AEP.

#### CHAPTER IV

### THE BIOSYNTHESIS OF THE PHOSPHONATE (C-P) BOND IN TETRAHYMENA

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## CHAPTER IV. THE BIOSYNTHESIS OF THE PHOSPHONATE (C-P)

### BOND IN TETRAHYMENA

#### Summary

1. A number of schemes for the formation of the C-P bond in Tetrahymena have been postulated and their feasibility examined experimentally.
2. Phosphite, which is frequently used in the chemical synthesis of phosphonates, was not detected in Tetrahymena.
3. A variety of  $^{14}\text{C}$ -compounds were tested as precursors for the AEP carbon skeleton.  $[6\text{-}^{14}\text{C}]\text{Glucose}$  and  $[1\text{-}^{14}\text{C}]\text{-glucose}$  were incorporated into AEP at higher degree than pyruvate, acetate as well as seven amino acid tested. In spite of the structural similarity, phosphatidylethanolamine and its phosphonic analogue, "AEP-cephalin", were shown to have different pathways of formation.
4. Attempts to demonstrate the synthesis of phosphonate by Tetrahymena preparations in vitro were unsuccessful.
5. A scheme of C-P bond formation through condensation between phosphoenolpyruvate and phosphatidic acid or its derivative has been offered and the possible mechanism of the reaction was discussed.

## 1. Introduction

The distribution in nature of phosphonates, notably of 2-aminoethylphosphonate (AEP), has been studied in several laboratories in recent years. However, the mechanism of C-P bond biosynthesis has proved rather elusive, and only limited experimental results dealing with this problem (Rosenberg, 1964) and some theoretical considerations (Segal, 1965) have so far been reported. Rosenberg (1964) showed that, when Tetrahymena was incubated in the presence of [ $^{32}\text{P}$ ]orthophosphate, the specific radioactivity of [ $^{32}\text{P}$ ]AEP in the cells was highest in the lipid-bound fraction which suggested that the site of C-P bond formation in Tetrahymena was a lipid molecule. These results have now been confirmed and enlarged. As shown in Chapter II, the saponifiable lipid of Tetrahymena acquired [ $^{32}\text{P}$ ]-label at a higher rate than non-saponifiable lipid. The rapidly labelled lipid was identified as AEP-cephalin.

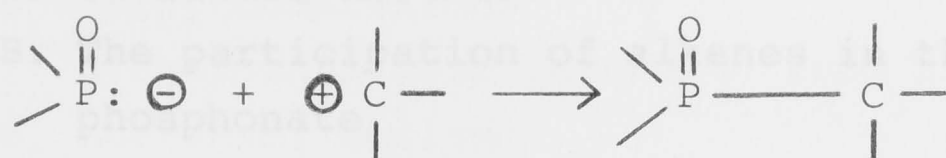
Although the biosynthesis of such a lipid through the cytidine derivative of AEP is possible, and the enzymes for this pathway have been shown to exist in Tetrahymena (Chapter III), the labelling pattern precludes this as the main biosynthetic route.

Segal (1965) postulated the possible formation of the C-P bond through a phosphoramidic rearrangement of phosphatidylethanolamine, but no experimental evidence was given.

This chapter is concerned with the mechanism of C-P bond formation in Tetrahymena. The work involved the use of compounds labelled with  $^{32}\text{P}$  as well as with  $^{14}\text{C}$  as potential precursors of the lipid-bound AEP.

A consideration of the known chemical reactions that lead to phosphonate synthesis suggests the condensation

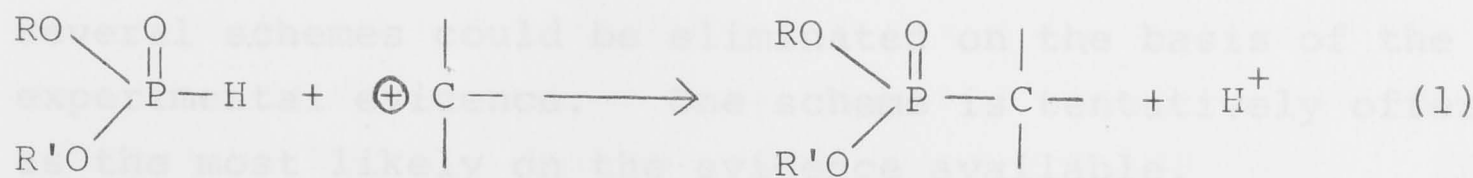
between an electrophilic carbon atom and a nucleophilic phosphorus atom (phosphite) as a possible mechanism of C-P bond formation. This can be represented as follows:



In the experiments described in this chapter, a number of hypothetical reactions, involving known metabolites, have been tested as the possible routes of C-P bond biosynthesis. These are:

A. AEP biosynthesis involving phosphite or its ester.

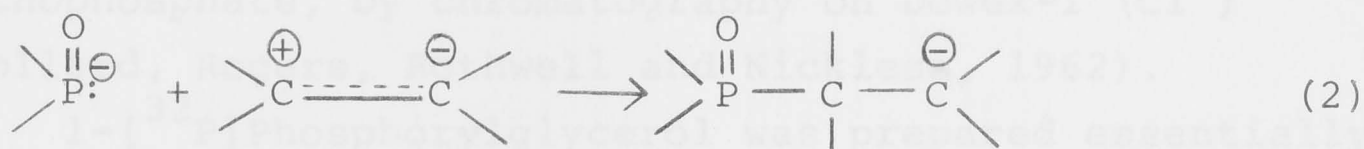
Phosphite esters play an important role in the chemical synthesis of phosphonates (cf. Chapter I), but very little is known about the natural occurrence of orthophosphite or of its participation in biological systems. Tsubota (1960) reported that Clostridium butyricum reduced orthophosphate to phosphite and to phosphine under anaerobic conditions. Horiguchi (1966) reported that, Clostridium butyricum, when grown in a medium in which part of the orthophosphate was replaced by metaphosphite, produced higher amounts of phosphonate. It was suggested that a reduced form of phosphate may be the precursor of phosphonate in C. butyricum. Therefore, phosphite (or its ester) could be one of the possible precursors of phosphonate. A possible scheme for the C-P bond formation may be represented as:



Since this scheme involves the reduction of ortho-phosphate (or of its ester) to the corresponding phosphite, the latter may be detectable by tracer methods even if present in minute amounts.

B. The participation of alkenes in the formation of phosphonate

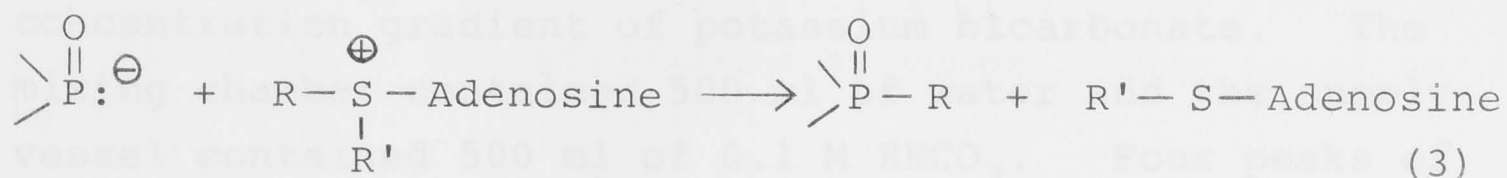
Nucleophilic attack of phosphite on alkenes can give rise to phosphonates as shown in equation (2):



Fumarate, a naturally occurring alkene, has been reported to react with phosphite by this mechanism (Hindersinn and Ludington, 1965).

C. Alkylation of phosphorus by an alkylsulphonium compound

An example of such a compound is S-adenosylmethionine which is a biological methylating agent.



An analogous reaction involving S-adenosylcystathionine could be visualized for the synthesis of AEP, although S-adenosylcystathionine is not known to occur in nature.

A number of experiments have been carried out to examine the hypothetical schemes suggested above, and several schemes could be eliminated on the basis of the experimental evidence. One scheme is tentatively offered as the most likely on the evidence available.



## 2. Materials and Methods

### A. Materials

[<sup>32</sup>P]Orthophosphate was purchased from the Australian Atomic Energy Commission.

[<sup>14</sup>C]Compounds were purchased from the Radiochemical Centre, Amersham, England.

[<sup>32</sup>P]Phosphite was purchased from the Radiochemical Centre, and was purified, to remove contaminating [<sup>32</sup>P]-orthophosphate, by chromatography on Dowex-1 (Cl<sup>-</sup>) (Pollard, Rogers, Rothwell and Nickless, 1962).

1-[<sup>32</sup>P]Phosphorylglycerol was prepared essentially as described by Possmayer and Strickland (1967) with a slight modification, as follows: one mmole of 3-chloropropane-1,2-diol dissolved in 2.5 ml distilled water was added to 1 mmole of [<sup>32</sup>P]Na<sub>3</sub>PO<sub>4</sub> containing 5 mC of radioactivity. The solution was incubated at 37° overnight. The mixture was passed through a column (2.5 cm x 35 cm) of Dowex-1 (bicarbonate) and was followed with 150 ml of water. The column was then eluted with an increasing concentration gradient of potassium bicarbonate. The mixing chamber contained 500 ml of water and the supply vessel contained 500 ml of 0.1 M KHCO<sub>3</sub>. Four peaks of radioactivity emerged and chromatographic examination of the eluate showed the bulk of the 1-phosphorylglycerol to be in peak 3, with a small portion overlapping into peak 4. The fractions comprising peak 3 were pooled and bicarbonate was removed by the addition of Dowex-50 (H<sup>+</sup>). The resin was then filtered off and the filtrate was adjusted to pH 8.0 with NaOH and taken to dryness. The residue was dissolved in 4 ml of water and re-chromatographed on Dowex-1 (bicarbonate) as described above. One symmetrical radioactive peak emerged. After the removal of bicarbonate, the material was subjected to paper chroma-

tography with solvent system II (see "Methods", below) followed by autoradiography. No contamination of [ $^{32}\text{P}$ ]-orthophosphate was found, and the material behaved as authentic 1-phosphorylglycerol.

#### B. Methods

Radioactivity of  $^{14}\text{C}$  was measured with a liquid Scintillation Spectrometer Model 314-DC (Packard Instrument Co. Inc.). Two kinds of scintillator were used depending on the nature of the samples:

- (a) Dioxane scintillator (Bray, 1960)
- (b) Toluene scintillator (Hayes, 1963).

Radioactivity in lipids from incubation mixtures was assayed according to the method described by Carter and Kennedy (1966), except that the "toluene" scintillator was used.

Radioactivity of [ $^{14}\text{C}$ ]AEP on paper was measured as follows: the chromatogram was lightly stained by dipping in 0.02% (w/v) ninhydrin in acetone and heating at  $80^\circ$  for 10 min. The AEP area was cut out, placed in a vial containing 5 ml of "toluene" scintillator and the radioactivity was measured. The efficiency of counting was found to be about 45%.

[ $^{32}\text{P}$ ]-labelled acidic lipid from Tetrahymena was prepared as follows: to 300 ml of a culture (17 h. of age) in the "complex" medium was added 10  $\mu\text{C}$  of carrier-free [ $^{32}\text{P}$ ]orthophosphate. After incubating at  $35^\circ$  for 2 h. the cells were harvested and the lipid was extracted as usual (cf. "Methods", Chapter II). Acidic lipids were isolated from the lipid mixture by chromatography on DEAE-cellulose (Rouser et al., 1963). About 4 mg of acidic lipids with 120,000 cpm of [ $^{32}\text{P}$ ] was obtained.

The uptake of  $^{32}\text{P}$  by Tetrahymena was measured by rapid filtration on membrane filters (Millipore) of samples

of cell suspensions in media containing  $^{32}\text{P}$ . The membrane was then washed with two 3 ml aliquots of 0.9% (w/v) NaCl, dried and pressed into a planchette. These were counted on a Nuclear Chicago Gas-flow Counter.

Detection of phosphate compounds on chromatograms was carried out by the method described by Hanes and Isherwood (1949).

Amino acid analysis was carried out in a Beckman Amino Acid Analyser (Model 120 B) fitted with a Nuclear Chicago Liquid Scintillation Counter (Model 720).

Chromatography on Whatman 3 MM paper was carried out using the following solvent systems:

I. Water-saturated phenol, acetic acid, ethanol (100:10:12, by vol.).

II. Isopropanol, ammonium hydroxide (28%), water (7:1:2, by vol.).

Other analytical methods referred to in this chapter have been described in detail in previous chapters.

### 3. Results

#### A. A search for phosphite in Tetrahymena

In support of the hypothetical scheme (1) shown in the Introduction, attempts were made to demonstrate the presence of phosphite in Tetrahymena.

Log phase Tetrahymena were labelled with 2  $\mu\text{C}$  of [ $^{32}\text{P}$ ]orthophosphate, and the cells (2 g of packed pellet) were hydrolyzed under "drastic" conditions (cf. "Methods", Chapter II). The hydrolysate was filtered through glass paper and taken to dryness. The residue was dissolved in a small amount of water, passed into a column (1.5 cm x 7 cm) of Dowex-1 (acetate) and washed through with 100 ml of 5% (v/v) acetic acid. The column was then eluted with 70 ml of 2N HCl (2N HCl removes both orthophosphate



and phosphite from the column) and the eluate was taken to dryness. The residue was dissolved in a small amount of water and subjected to paper electrophoresis, followed by autoradiography. Only radioactive orthophosphate could be detected. When in an identical experiment [ $^{32}\text{P}$ ]phosphite mixed with Tetrahymena homogenate was hydrolysed, and the hydrolysate examined as described above, [ $^{32}\text{P}$ ]phosphite was the only radioactive material found. The presence of phosphite in Tetrahymena could thus not be demonstrated.

The utilization of phosphite by Tetrahymena was examined by testing its uptake by the cells. It was found (Fig. IV.1) that the cells failed to take up phosphite under conditions where rapid uptake of orthophosphate took place. Thus, phosphite could not be shown to be a precursor of AEP in Tetrahymena.

B. A survey of potential precursors of the carbon skeleton of AEP

Various [ $^{14}\text{C}$ ]-labelled compounds have been tested as precursors of the carbon skeleton of AEP in Tetrahymena. Log phase cells (0.5 g of wet packed cells) were suspended in 20 ml of a solution containing 40 mM NaCl, 1 mM  $\text{MgCl}_2$  and one-tenth concentration of the "defined" medium (cf. "Methods", Chapter II). To the cell suspension was added 1  $\mu\text{C}$  of the [ $^{14}\text{C}$ ]-compound (specific radioactivity 3  $\mu\text{C}$  to 30  $\mu\text{C}$  per  $\mu\text{mole}$ ). The suspensions were shaken on a water bath at  $35^\circ$  for 3 hours and the uptake of radioactivity by the cells was measured by following the decrease of radioactivity in the medium during incubation. After incubation, 1 ml of 100% (w/v) trichloroacetic acid was added to the flask and the precipitate was collected by centrifugation. The precipitate was washed once with 5% (w/v) trichloroacetic acid and the lipid was extracted



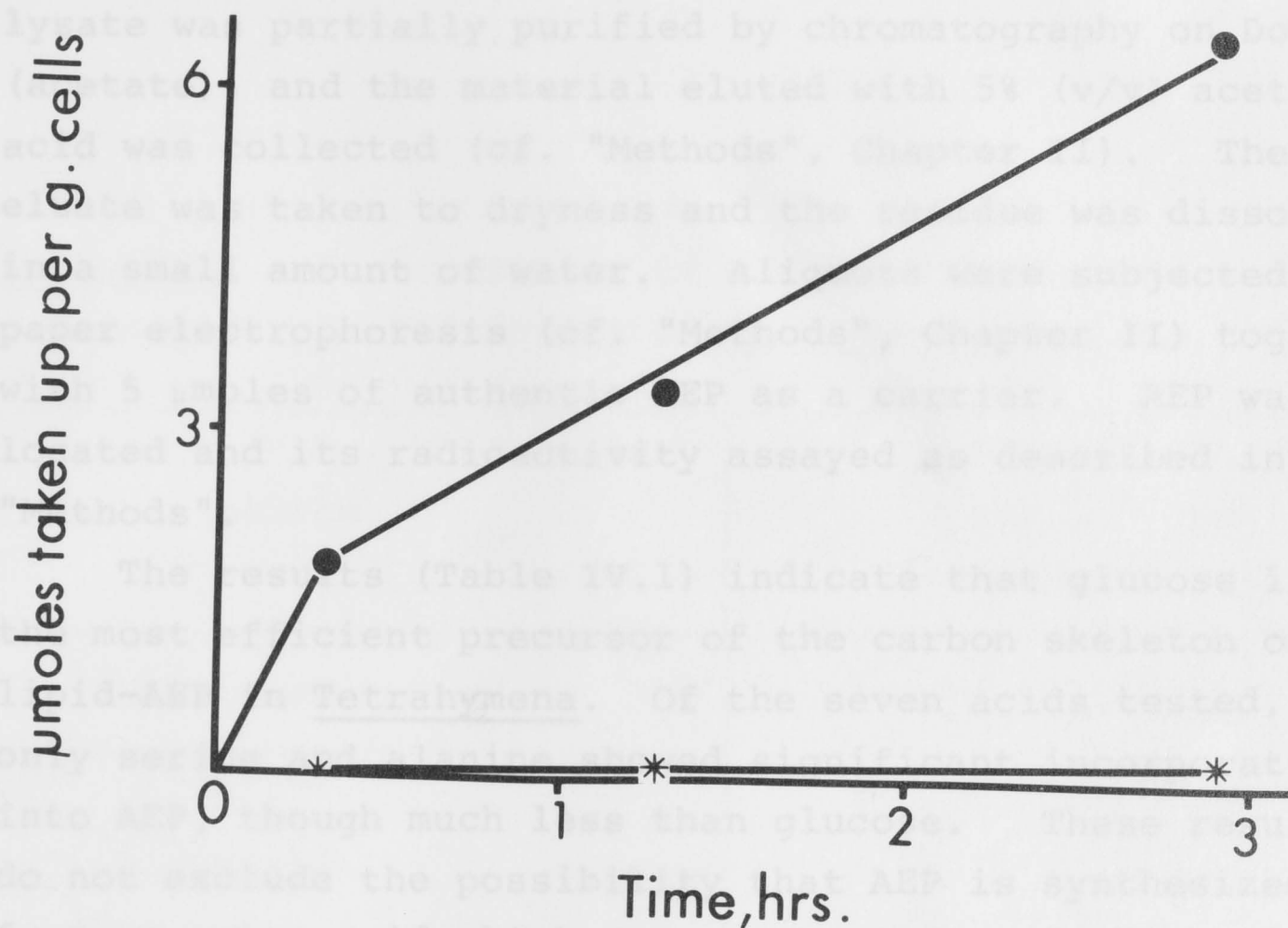


Fig. IV.1. The uptake of phosphate and phosphite by Tetrahymena. Each flask contained 25 ml of "defined" medium with 0.2 mM [<sup>32</sup>P]orthophosphate or [<sup>32</sup>P]phosphite (1.2 μC each) and 0.4 g of wet packed cells. At time intervals, 0.5 ml of the suspension was withdrawn and rapidly filtered on a membrane filter (Millipore, see "Methods") and the radioactivity of [<sup>32</sup>P] in the cells was measured.

● ————— ● [<sup>32</sup>P]orthophosphate

\* ————— \* [<sup>32</sup>P]phosphite

as described (cf. "Methods", Chapter II). The lipid was hydrolyzed under "drastic" conditions. AEP in the hydrolysate was partially purified by chromatography on Dowex-1 (acetate), and the material eluted with 5% (v/v) acetic acid was collected (cf. "Methods", Chapter II). The eluate was taken to dryness and the residue was dissolved in a small amount of water. Aliquots were subjected to paper electrophoresis (cf. "Methods", Chapter II) together with 5  $\mu$ moles of authentic AEP as a carrier. AEP was located and its radioactivity assayed as described in "Methods".

The results (Table IV.1) indicate that glucose is the most efficient precursor of the carbon skeleton of lipid-AEP in Tetrahymena. Of the seven acids tested, only serine and alanine showed significant incorporation into AEP, though much less than glucose. These results do not exclude the possibility that AEP is synthesized from an amino acid which was not tested, and which, in turn, has glucose as its precursor. This possibility has now been examined.

Log phase Tetrahymena (2.5 g of wet packed cells) were suspended in 50 ml of the "complex" medium containing 1 mM and 5  $\mu$ C of [6-<sup>14</sup>C]glucose. The suspension was incubated at 35° for 4 hours, during which time 70% of the added radioactive material was taken up by the cells. The cell suspension was cooled in an ice bath for 10 min. and the cells were collected by centrifugation. The acid-soluble fraction of the cells was prepared as described (cf. "Methods", Chapter II). The acid-soluble fraction was then applied to a column (1.5 cm x 6 cm) of Dowex-50 (H<sup>+</sup>), the column was washed with 100 ml of water and eluted with 30 ml of 2.5 N ammonium hydroxide. The original water wash and the eluate were each taken to

Table IV.1. Incorporation of carbon from various compounds into the AEP moiety of Tetrahymena lipids

Carbon source	cpm in [ $^{14}\text{C}$ ]AEP per $10^6$ cpm taken up by cells
[1- $^{14}\text{C}$ ]glucose	1,100
[6- $^{14}\text{C}$ ]glucose	1,200
[2- $^{14}\text{C}$ ]acetate	< 100
[3- $^{14}\text{C}$ ]pyruvate	200
[3- $^{14}\text{C}$ ]cysteine	< 100
[3- $^{14}\text{C}$ ]serine	200
[U- $^{14}\text{C}$ ]alanine	200
[U- $^{14}\text{C}$ ]aspartate	< 100
[U- $^{14}\text{C}$ ]glutamate	< 100
[2- $^{14}\text{C}$ ]ethanolamine	< 100
[ $^{14}\text{C}$ ]K $_2$ CO $_3$	< 100
[ $^{14}\text{CH}_3$ ]methionine	< 100
[2- $^{14}\text{C}$ ]glycine	< 100

dryness. The residue from the water wash was dissolved in 10 ml of 5% (v/v) acetic acid and treated with 40 mg of Norit-A charcoal at 0° for 3 min. then left at 2° for 30 min. The charcoal was filtered off, the filtrate was taken to dryness and the residue was dissolved in 2 ml of water. The solution was passed into a column (1.5 cm x 3 cm) of Dowex-1 (acetate) and the column washed with 30 ml of water. The water effluent was discarded. The column was then eluted with 20 ml of 5% (v/v) acetic acid and the eluate was taken to dryness. This residue was combined with the residue obtained from the ammonium hydroxide eluate of Dowex-50 (combined weight, 57.2 mg; combined radioactivity,  $9 \times 10^4$  cpm). An aliquot of this mixture was used for the analysis of the amino acids and their radioactivities.

As could be expected, the three amino acids directly derived from the keto acids of the glycolytic and tri-carboxylic acid pathways, namely, alanine, glutamate and aspartate, contained the highest amount of the label (Fig. IV.2). However, as shown above (Table IV.1), none of these amino acids are efficient as carbon donors in the synthesis of AEP in Tetrahymena. The comparatively high incorporation of label from  $[6-^{14}\text{C}]$ glucose and  $[1-^{14}\text{C}]$ -glucose, and the low degree of incorporation from  $[3-^{14}\text{C}]$ -pyruvate or  $[2-^{14}\text{C}]$ acetate into AEP suggested one of the intermediates of the glycolytic pathway as the precursor of AEP in Tetrahymena.

$[1-^{14}\text{C}]$ Glycerol was also tested in the same experiment. However, Tetrahymena took up glycerol very slowly (the uptake rate was about 1/9,000 that of glucose on a molar basis over a 3-hour incubation). Furthermore, the incorporation of the label from glycerol into AEP was very low, so that proper comparison with glucose could not be



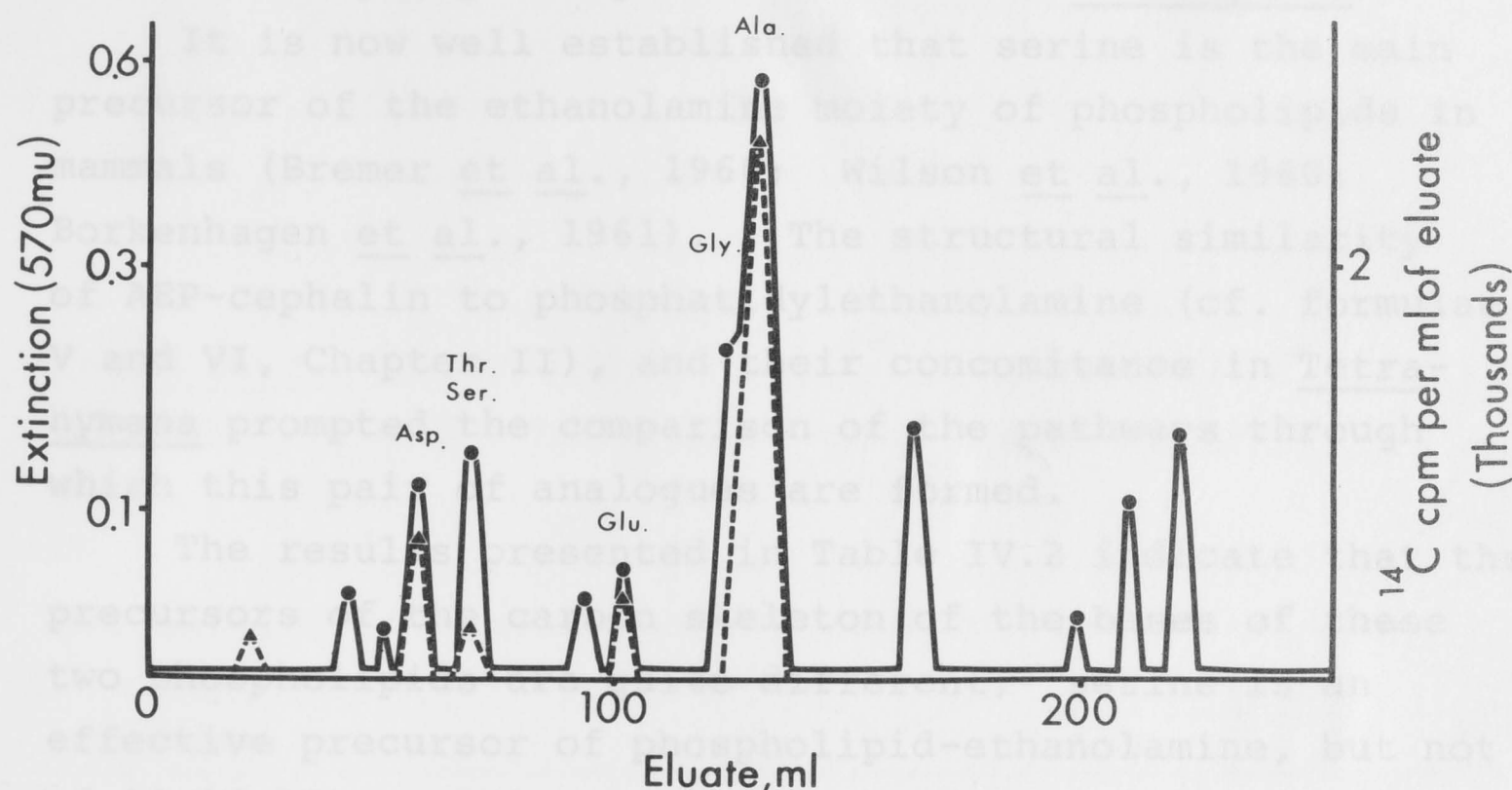


Fig. IV.2. The distribution of radioactivity in the free amino acids from *Tetrahymena* shaken in the presence of 5  $\mu$ C of [6-<sup>14</sup>C]glucose for 4 hours. The free amino acids in the cells were separated and partially purified (see text). An aliquot of the sample was passed through an amino acid analyzer fitted with a liquid scintillation counter (cf. "Methods"). The distribution of amino acids as well as their radioactivities in the sample were analyzed.

● — ● Color density after ninhydrin reaction

▲ - - - ▲ <sup>14</sup>C Radioactivity

made.

C. The incorporation of [ $^{14}\text{C}$ ]serine into AEP-cephalin and phosphatidylethanolamine in Tetrahymena

It is now well established that serine is the main precursor of the ethanolamine moiety of phospholipids in mammals (Bremer et al., 1960; Wilson et al., 1960; Borkenhagen et al., 1961). The structural similarity of AEP-cephalin to phosphatidylethanolamine (cf. formulae V and VI, Chapter II), and their concomitance in Tetrahymena prompted the comparison of the pathways through which this pair of analogues are formed.

The results presented in Table IV.2 indicate that the precursors of the carbon skeleton of the bases of these two phospholipids are quite different; serine is an effective precursor of phospholipid-ethanolamine, but not of lipid-AEP. This result precludes phosphatidylethanolamine as a precursor of AEP-cephalin, as suggested by Segal (1965), and indicates that the synthetic pathways of AEP-cephalin and phosphatidylethanolamine are different.

The  $\text{C}_3$ -unit ( $-\text{S}-\text{CH}_2\overset{\text{NH}_2}{\underset{|}{\text{CH}}}\text{COOH}$ ) of cystathionine is derived from serine (Binkley, 1951; Selim and Greenberg, 1959) or cysteine (Rowbury and Woods, 1964; Kaplan and Flavin, 1965). The low degree of incorporation of carbon from either serine or cysteine into AEP compared with that from glucose rendered improbable the origin of AEP from cystathionine. Therefore the possibility of C-P bond formation through scheme (3) (see "Introduction") is eliminated.

The demonstrated high degree of incorporation of [ $^{14}\text{C}$ ]serine into phospholipid-ethanolamine suggested that the exchange reaction (shown below) demonstrated by Borkenhagen et al. (1961) in rat liver, occurs also in

Tetrahymena

Ethanolamine

Phosphatidylserine

Serine

Phosphatidylethanolamine

CO<sub>2</sub>D. Attempts at the biosynthesis of AEP by Tetrahymena

preparations in vitro

Table IV.2. Incorporation of [<sup>14</sup>C]glucose and [<sup>14</sup>C]serine into the AEP and ethanolamine moieties of Tetrahymena lipid

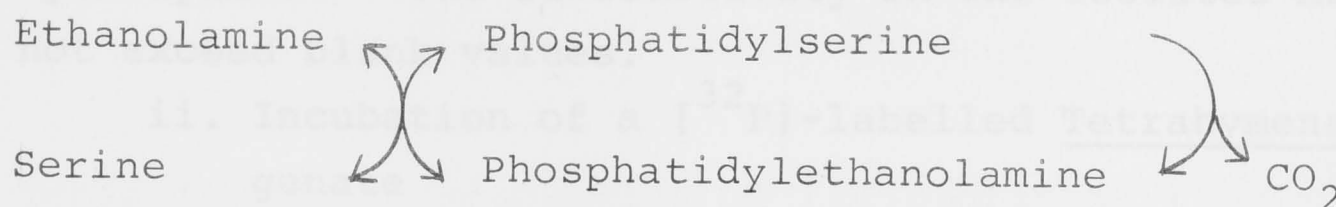
For details of experimental procedure, see Table IV.1.

1. Incubation of Tetrahymena cell-free homogenatewith [<sup>32</sup>P]acidic lipid

Carbon source	Incorporation of <sup>14</sup> C in cpm per 10 <sup>6</sup> cpm taken up by the cells	
	Lipid-AEP	Lipid-ethanolamine
[6- <sup>14</sup> C]glucose	1,100	310
[3- <sup>14</sup> C]serine	50	73,500

could be the precursor of the carbon moiety of AEP. For this reason, phosphoenolpyruvate was added to the reaction mixture.

A cell-free homogenate from Tetrahymena was incubated with [<sup>32</sup>P]acidic lipids of Tetrahymena (cf. "Methods") (2 mg, 50,000 cpm) in the presence of ATP (1 mM), K-phosphoenolpyruvate (2 mM), thiamine pyrophosphate (0.2 mM) and Tris-HCl (50 mM, pH 7.4). After incubation (with shaking) at 35° for 3 hours, the mixture was hydrolyzed

Tetrahymena.

D. Attempts at the biosynthesis of AEP by Tetrahymena preparations in vitro

Some attempts were made to achieve the synthesis of AEP using cell-free systems from Tetrahymena. Unfortunately, no AEP synthesis (or C-P bond synthesis) occurred in the systems employed. However, some of those experiments are described below.

i. Incubation of Tetrahymena cell-free homogenate with [<sup>32</sup>P]acidic lipid.

Previous results (cf. Chapter II) suggest that AEP-cephalin is the site where C-P bond is synthesized in Tetrahymena. The phosphorus precursor of this compound would be phosphatidic acid or its derivatives. In order to investigate this possibility in vitro, [<sup>32</sup>P]acidic lipids from Tetrahymena, which contained mainly phosphatidic acid (Rouser et al., 1963), were prepared (cf. "Methods"). Considering the previous results (Table IV.1) and scheme (2) (see "Introduction"), phosphoenolpyruvate could be the precursor of the carbon moiety of AEP. For this reason, phosphoenolpyruvate was added to the reaction mixture.

A cell-free homogenate from Tetrahymena was incubated with [<sup>32</sup>P]acidic lipids of Tetrahymena (cf. "Methods") (2 mg, 60,000 cpm) in the presence of ATP (1 mM), K-phosphoenolpyruvate (2 mM), thiamine pyrophosphate (0.2 mM) and Tris-HCl (50 mM, pH 7.4). After incubation (with shaking) at 35° for 3 hours, the mixture was hydrolysed



under "drastic" conditions and [ $^{32}\text{P}$ ]AEP was assayed in the hydrolysate. The radioactivity in the isolated AEP did not exceed blank values.

ii. Incubation of a [ $^{32}\text{P}$ ]-labelled Tetrahymena homogenate

Log phase cells (2.5 g wet packed) were suspended in the "defined" medium containing 0.2 mM and 10  $\mu\text{C}$  of [ $^{32}\text{P}$ ]-orthophosphate. After one hour's incubation at 35°, the cells took up 3  $\mu\text{C}$  of [ $^{32}\text{P}$ ]orthophosphate. The cells were collected and a cell-free homogenate was prepared as usual (cf. "Methods", Chapter III). The [ $^{32}\text{P}$ ]-labelled homogenate was then incubated in the presence of phosphoenolpyruvate (2 mM), ATP, (3 mM) and thiamine pyrophosphate (0.2 mM). Examination of the hydrolysate of the incubation mixture did not show any increase in radioactive AEP during the second incubation.

iii. Incorporation of [ $^{14}\text{C}$ ]ethanolamine into the lipid fraction of Tetrahymena in vitro

Horiguchi (1966) reported that the presence of phosphate increased the incorporation of [ $^{14}\text{C}$ ]ethanolamine into AEP by a cell-free homogenate from Tetrahymena. Since no evidence was offered in support of this claim, the final conclusion must await further experimental proof. It is, however, clear from the present results (cf. Chapter II) that, if ethanolamine were incorporated into AEP, it would be found in the lipid. The incorporation of ethanolamine into the lipid fraction of Tetrahymena in vitro, was therefore examined and the labelling of the ethanolamine and AEP moieties of the phospholipid were compared.

It was found (Table IV.3) that ethanolamine was incorporated into the lipid fraction of Tetrahymena cell-free homogenate in the absence of added CTP. The decrease of

77

incorporation of label by the addition of "cold" phosphoryl-ethanolamine (PE) was only about one-fourth of that expected if the latter was an intermediate (Kennedy and Weiss, 1956). This suggests that the exchange reaction (shown in Table IV.3) is not the case. At the same time, no incorporation of label from ethanolamine into lipid-AEP was observed.

Table IV.3. Incorporation of radioactivity from [ $^{14}\text{C}$ ]-ethanolamine into lipid-ethanolamine and lipid-bound AEP in Tetrahymena in vitro

The incubation mixture contained Tetrahymena cell-free homogenate (30 mg protein), Tris-HCl (pH 7.4, 50 mM), [ $2\text{-}^{14}\text{C}$ ]ethanolamine (1  $\mu\text{C}$ , specific radioactivity, 3.6  $\mu\text{C}$  per  $\mu\text{mole}$ ) and additions as indicated. Final volume was 1 ml. Control tube was prepared by adding [ $^{14}\text{C}$ ]-ethanolamine after incubation.

	Radioactivity (cpm) in lipid	
	Ethanolamine	AEP
Complete system	58,450	26
Addition of 2.5 $\mu\text{moles}$ PE	21,950	26
Control	150	25

#### 4. Discussion

The ability of Clostridium butyricum to reduce phosphate to phosphite (Taubert, 1960) and the claim that phosphonate levels increased in Cl. butyricum when phosphite was present in the medium (Uriguchi, 1966) suggested a possible involvement of phosphite in phospho-

incorporation of label by the addition of "cold" phosphoryl-ethanolamine (PE) was only about one-fourth of that expected if the latter was an intermediate (Kennedy and Weiss, 1956). This suggests that the exchange reaction (shown above) of Borkenhagen et al. (1961) is largely responsible for the incorporation of free ethanolamine into cephalin of Tetrahymena and that it does not involve CDPE. At the same time, no incorporation of label from ethanolamine into lipid-AEP could be detected in the same system.

iv. Incorporation of  $^{32}\text{P}$  from [ $^{32}\text{P}$ ]-1-phosphoryl-glycerol

Previous results (cf. Chapter II) pointed to phosphatidic acid or its derivative as one of the likeliest precursors of the phosphorus moiety of AEP in Tetrahymena. [ $^{32}\text{P}$ ]-1-Phosphorylglycerol was therefore tested as a precursor of AEP-phosphorus. Unfortunately, under the usual incubation conditions and even following phosphate-starvation, whole cells failed to take up 1-phosphorylglycerol.

Incubation of a cell-free homogenate of Tetrahymena with [ $^{32}\text{P}$ ]-1-phosphorylglycerol in reaction systems described by Possmayer and Strickland (1967) or by Paulus and Kennedy (1960) did not lead to any significant incorporation of the 1-phosphorylglycerol into the lipid fraction of the homogenate. Thus the incorporation of 1-phosphorylglycerol into lipid-AEP could not be demonstrated.

#### 4. Discussion

The ability of Clostridium butyricum to reduce phosphate to phosphite or phosphine (Tsubota, 1960) and the claim that phosphonate levels increased in Cl. butyricum when phosphite was present in the medium (Horiguchi, 1966) suggested a possible involvement of phosphite in phospho-

nate biosynthesis. Although the presence of phosphite could not be demonstrated in Tetrahymena, and cells failed to take up phosphite, its participation in AEP biosynthesis cannot be completely excluded. Thus a transient intermediate containing phosphorus in a reduced form of phosphate may be present in Tetrahymena and may react with a carbon precursor to give rise to phosphonate.

The results presented above are consistent with the following: (1) the only phosphonates found in Tetrahymena are AEP and small amounts of 2-phosphonoalanine (Kittredge and Hughes, 1964); (2) both [6-<sup>14</sup>C] and [1-<sup>14</sup>C]glucose are better precursors of the carbon of AEP than pyruvate or acetate (Table IV.1); and (3) a free amino acid does not appear to be the direct precursor of AEP.

The amino group of the AEP molecule must thus arise by transamination after the formation of the C-P bond.

The precursor of the carbon moiety of AEP must therefore satisfy the following conditions:

- (1) It should be an intermediate of glycolysis, or a compound derived from a glycolytic intermediate.
- (2) It should be capable of accepting an amino group.
- (3) It must present a site for nucleophilic attack by monomeric metaphosphate to form the C-P bond (ethylene or some other unsaturated form).

It appears that phosphoenolpyruvate is the only known glycolytic intermediate that meets all three requirements.

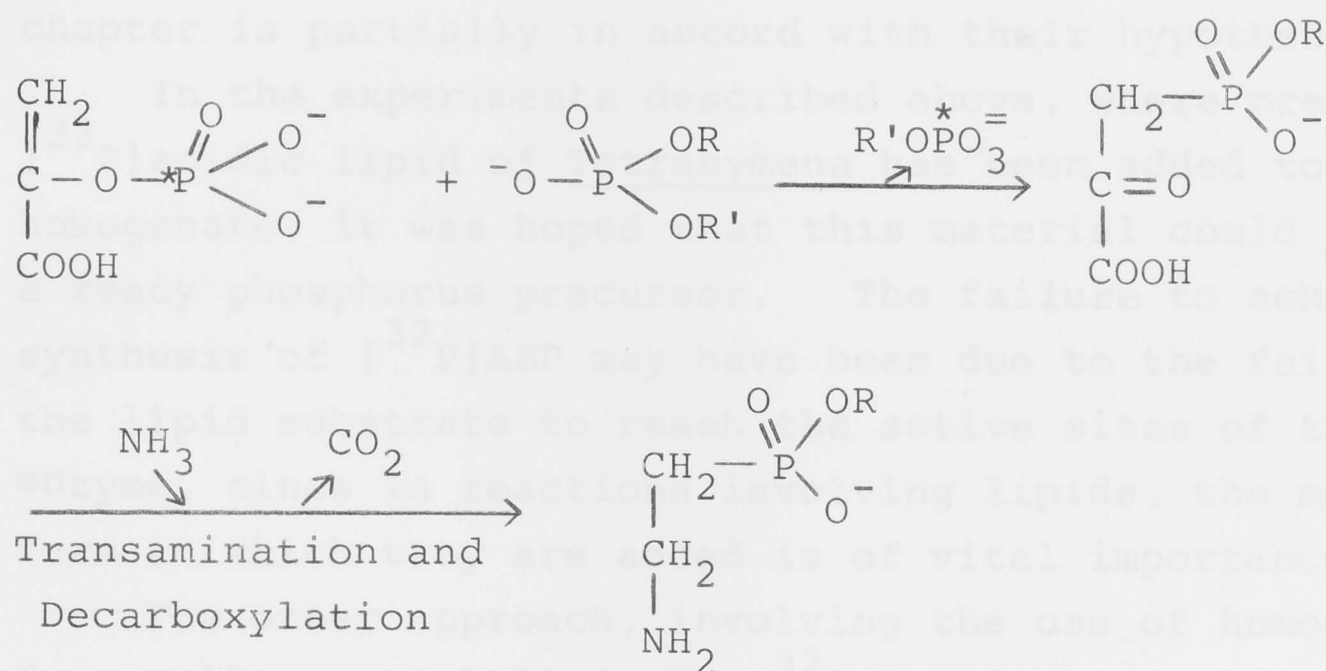
Of the known facts concerning the precursor of the phosphorus of AEP, two appear to be most relevant:

- (1) AEP-phosphorus is derived from phospholipid phosphorus, and
- (2) phosphatidylethanolamine is not a precursor of AEP.



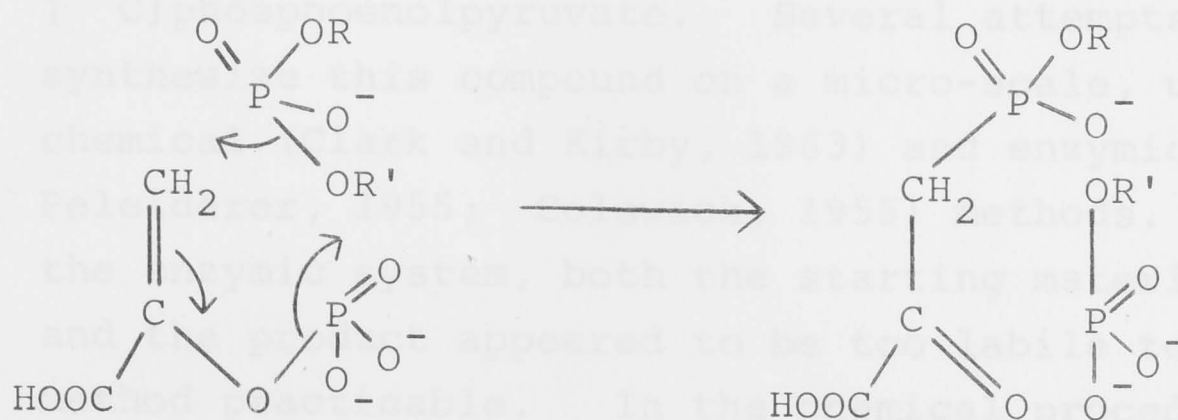
Hence in the proposed mechanism the phosphorus donor could be phosphatidic acid. However, it has been established (cf. Chapter I) that diesterified phosphate is more reactive in the type of reaction represented below. It is therefore suggested that some type of diester derived from phosphatidate participates in Tetrahymena.

The following scheme of C-P bond formation in Tetrahymena is suggested on the basis of the results presented in this thesis:



where R is diglyceride, the nature of R' is not known (see below).

The suggested electron shift in the above scheme would be:



In the above scheme, where R is a diglyceride, R' may be cytidine monophosphate, so that the reactant is CDP-diglyceride; or it may be polyglyceride (reactant : cardiolipin).

Lehmann and Benson (1964) were able to synthesize sulfoacetic acid from sulphite and phosphoenolpyruvate and they suggested that phosphoryl transfer from ATP to enol acceptors may also be involved in the biosynthesis of phosphonic acid. The mechanism suggested in this chapter is partially in accord with their hypothesis.

In the experiments described above, where pre-labelled [ $^{32}\text{P}$ ]acidic lipid of Tetrahymena has been added to the homogenate, it was hoped that this material could provide a ready phosphorus precursor. The failure to achieve synthesis of [ $^{32}\text{P}$ ]AEP may have been due to the failure of the lipid substrate to reach the active sites of the enzyme, since in reactions involving lipids, the micellar form in which they are added is of vital importance.

The other approach, involving the use of homogenates from cells pre-labelled with  $^{32}\text{P}$  in vivo, was tried to avoid this difficulty. However, even in this case no increase in [ $^{32}\text{P}$ ]AEP was observed. Addition of unlabelled phosphoenolpyruvate to this system was also without success.

The obvious substrate to test in this pathway is [ $^{14}\text{C}$ ]phosphoenolpyruvate. Several attempts were made to synthesize this compound on a micro-scale, using both chemical (Clark and Kirby, 1963) and enzymic (Bücher and Peleiderer, 1955; Colowick, 1955) methods. However, in the enzymic system, both the starting material (pyruvate) and the product appeared to be too labile to make the method practicable. In the chemical procedure, the very small amounts handled resulted in the loss of the inter-

mediate (bromopyruvate) due to the smallest traces of water which could not be excluded (this is a lesser hazard with a larger scale preparation). The prohibitive cost of the isotopic starting material and the need of high specific activity material ruled out chemical synthesis on a convenient scale.

#### CHAPTER V

### THE BIOSYNTHESIS OF PHOSPHONATES IN THE SLOO *LEHMANNIA POIRIERI* : A COMPARATIVE STUDY

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CHAPTER V. THE BIOSYNTHESIS OF PHOSPHONATES IN THE SLUG  
*LEHMANNIA POIRIEI* : A COMPARATIVE STUDY

Summary

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CHAPTER V. THE BIOSYNTHESIS OF PHOSPHONATES IN THE SLUG  
LEHMANNIA POIRIERI : A COMPARATIVE STUDY

Summary

1. The existence of phosphonate in slugs has been confirmed and the compounds were identified as 2-aminoethylphosphonate (AEP) and its derivatives.
2.  $^{32}\text{P}$ -Incorporation studies showed that phosphonate in the acid-soluble fraction of the slug attained higher specific radioactivity than lipid or residual bound phosphonate. The bulk of the radioactive phosphonate in the acid-soluble fraction was identified as free AEP.
3. A number of  $^{14}\text{C}$ -compounds were tested as potential precursors of AEP in the slugs. Of the compounds tested, [ $^{14}\text{C}$ ]glucose showed highest incorporation into AEP, and this suggested that the mechanism of C-P bond formation in the slug may be similar to that in Tetrahymena.

2. Materials and Methods

2.1. Materials

The slugs (Lehmanna poirieri) were collected in garden throughout Canberra, Australia. They were kept in

## 1. Introduction

The occurrence of phosphonates in members of the phylum Mollusca is well documented.

Quin (1965) reported that the bivalves Mytilus edulis and Venus mercenaria contained phosphonate-compounds which were soluble in 70% ethanol. Hydrolysis of this fraction yielded AEP. Unidentified phosphonates were also detected in the tissue of the gastropod mollusc Busycon canaliculatum. Hori et al. (1964) reported the presence of ceramide aminoethylphosphonate in the shellfish Corbicula sandai as well as in five other species of shellfish. De Koning (1966) reported that AEP was present in the lipid fraction of the shellfish abalone (Haliotis midae). Horiguchi (1966) reported that the garden slugs (the strain was not named) contained phosphorus compounds which were resistant to acid hydrolysis.

The work described in this chapter deals with the identification of AEP in the garden slug, Lehmannia poirieri, and with the pattern of its biosynthesis by this organism in vivo. A comparison of C-P bond synthesis in the slug and in Tetrahymena has been made and the possible mechanism of C-P bond formation in the two organisms is discussed.

Although this thesis is primarily concerned with the metabolism of phosphonate in Tetrahymena, it was felt that the inclusion of this chapter was justified in order to point out a fundamental difference in the site of biosynthesis of the C-P bond in the slug from that in Tetrahymena.

## 2. Materials and Methods

### A. Materials

The slugs (Lehmannia poirieri) were collected in gardens throughout Canberra, Australia. They were kept in

the dark at 21°-22° in a wooden box containing moist leaf debris and grass.

[<sup>32</sup>P]Orthophosphate was purchased from the Australian Atomic Energy Commission.

[<sup>14</sup>C]Compounds were purchased from the Radiochemical Centre, Amersham, England.

#### B. Methods

Homogenates were prepared using a NELCO high-speed microblender.

The various analytical and other methods referred to in this chapter have been described in detail in Chapters II and IV.

Ascending paper chromatography on Whatman 3 MM paper was carried out in the following solvent systems:

- I. n-Butanol, acetic acid, water (4:1:5, by vol.), upper layer used.
- II. Water-saturated phenol.

### 3. Results

#### A. Detection of AEP in the slug

Two slugs, weighing 0.7 g each, were injected with a total of 10  $\mu$ C of carrier-free [<sup>32</sup>P]orthophosphate dorsally, through the body wall. After 24 hours, they were washed once with saline and quickly homogenized in 5 ml of water. Immediately, 5 ml of 10% (w/v) trichloroacetic acid was added to the homogenate and the mixture was stirred vigorously for 1 min. The acid-soluble, lipid and residual fractions were separated and each fraction was hydrolyzed under "drastic" conditions (6N HCl, 48 hours, 120°). The hydrolysates were rendered acid-free (cf. "Methods", Chapter II) and applied to columns of Dowex-1 (acetate). The columns were eluted consecutively with 5% (v/v) acetic acid and 2N HCl and the radioactivity



of the eluates were measured.

It was found (Table V.1) that a considerable amount of radioactivity was present in the acetic acid eluates from the hydrolysates of all three fractions. In the absence of phosphonates, orthophosphate is the only phosphorus compound present in such hydrolysates (see Chapter II). Furthermore, since AEP, but not orthophosphate, is removed from Dowex-1 (acetate) by 5% (v/v) acetic acid, these results strongly indicated the presence of AEP (or other aminoalkylphosphonates) in all three hydrolysates. This possibility was further investigated.

Samples of the acetic acid eluates from the resin were taken to dryness and the residue was taken up in a drop of water and applied to a Whatman 3 MM paper. The paper was subjected to electrophoresis followed by autoradiography (cf. "Methods", Chapter II). Ninhydrin-staining of the paper showed the presence of bands which migrated as AEP in each sample. Autoradiography showed only one radioactive band in each of the samples; these, in all three cases, corresponded to the ninhydrin-staining bands. These results again strongly indicated the presence of AEP as well as its de novo synthesis in the slug.

#### B. The location of AEP synthesis in the slug

The ability of the slug to synthesize AEP (or the C-P bond) has been tentatively demonstrated. In order to find the molecule (or molecules) in which the C-P bond was synthesized, the rates of AEP formation in different fractions were compared.

It was found (Fig. V.1) that incorporation of  $^{32}\text{P}$  into phosphonate was most rapid in the acid-soluble fraction. The residual fraction was next, the lipid fraction being the slowest. Although the residual fraction contained over 60% of the total phosphonate (Table V.2), its

Table V.1. Distribution of radioactivities in fractions of slugs 24 hours after injection of [ $^{32}\text{P}$ ]orthophosphate.

Each fraction was hydrolyzed and the hydrolysate was passed through Dowex-1 (acetate) which was eluted with 5% acetic acid followed by 2N HCl.

Fraction	Total $^{32}\text{P}$ radioactivity (cpm)	
	Acetic acid eluate	HCl eluate
Acid-soluble	7,400	286,000
Lipid	2,200	22,000
Residual	6,300	262,000

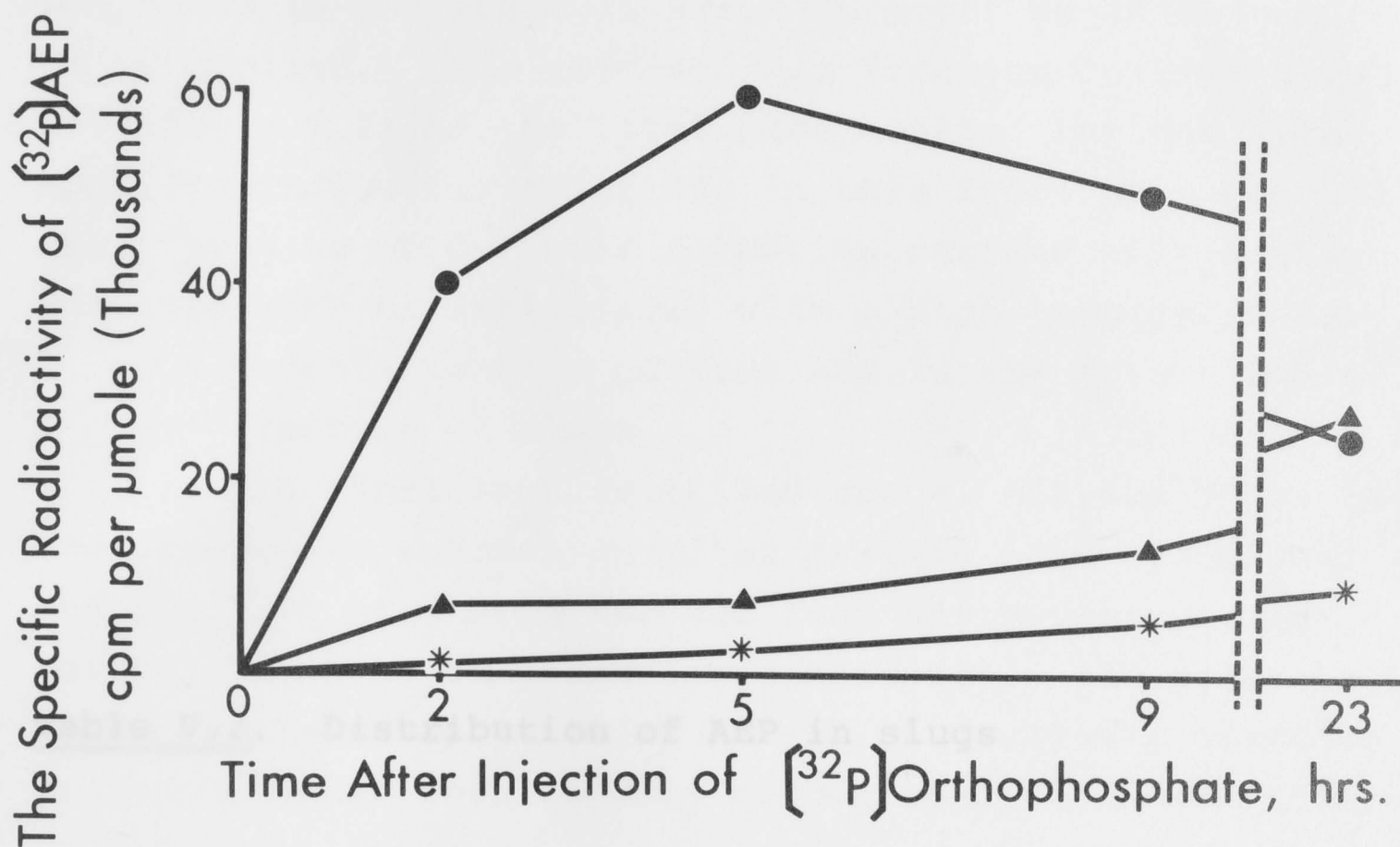


Fig. V.1. The time course of AEP labelling in the slug. Eleven slugs of an average weight 1.5 g, were each injected with 10  $\mu$ C carrier-free [ $^{32}$ P]-orthophosphate. At intervals shown, groups of animals numbering 2-3 were killed by homogenising with trichloroacetic acid and the specific radioactivity of the AEP-phosphorus in the various fractions was assayed using the methods described.

- ——— ● Acid-soluble fraction
- ▲ ——— ▲ Residual fraction
- \* ——— \* Lipid fraction

low specific radioactivity compared to the phosphonate in the acid-soluble fraction suggested that the phosphonate compounds in the residual fraction might be an end product of metabolism. The acid-soluble fraction contained only 15% (Table V.2) of the total phosphonate, but the high specific radioactivity of AEP in this fraction suggested that the site of C-P bond formation resides in a small molecule (AEP or derivative) with a high turnover rate.

#### C. Identification of free AEP in the acid-soluble fraction of slugs

In the experiment described above, AEP was found in the acid-soluble fraction after drastic acid hydrolysis and may thus have been derived from another small, but partially labile compound (or compounds). In order to

Table V.2. Distribution of AEP in slugs

Fraction	$\mu$ moles per g of wet tissue	% of total AEP
Acid-soluble	0.93	15.8
Lipid	1.37	23.3
Residual	3.57	60.9

(A-E, Fig. V.2) were hydrolyzed under "drastic" conditions and the distribution of the radioactivity in phosphonate as well as in orthophosphate in each hydrolysate was assayed.

It was found (Table V.3) that the bulk of the radioactive phosphonate was present in fraction "B". Fractions "C" and "D" contained only minor traces of phosphonate. Fraction "B" was further chromatographed on Dowex-1 (formate)



low specific radioactivity compared to the phosphonate in the acid-soluble fraction suggested that the phosphonate compounds in the residual fraction might be an end product of metabolism. The acid-soluble fraction contained only 15% (Table V.2) of the total phosphonate, but the high specific radioactivity of AEP in this fraction suggested that the site of C-P bond formation resides in a small molecule (AEP or derivative) with a high turnover rate.

#### C. Identification of free AEP in the acid-soluble fraction of slugs

In the experiment described above, AEP was found in the acid-soluble fraction after drastic acid hydrolysis and may thus have been derived from another small, but partially labile compound (or compounds). In order to identify the phosphonate(s) in the acid-soluble fraction, isolation under considerably milder conditions was undertaken.

Fourteen slugs (average weight 1.1 g each), were each injected with 20  $\mu$ C of carrier free [ $^{32}$ P]orthophosphate. They were all homogenized four hours later, and the acid-soluble material was extracted. The extract was rendered acid-free and taken to dryness, and the residue was taken up in a small volume of water and chromatographed on Dowex-50 ( $H^+$  form) and Dowex-1 (acetate form) (Fig. V.2).

Samples from each of the five fractions obtained (A-E, Fig. V.2) were hydrolyzed under "drastic" conditions and the distribution of the radioactivity in phosphonate as well as in orthophosphate in each hydrolysate was assayed.

It was found (Table V.3) that the bulk of the radioactive phosphonate was present in fraction "B". Fractions "C" and "D" contained only minor traces of phosphonate. Fraction "B" was further chromatographed on Dowex-1 (formate).

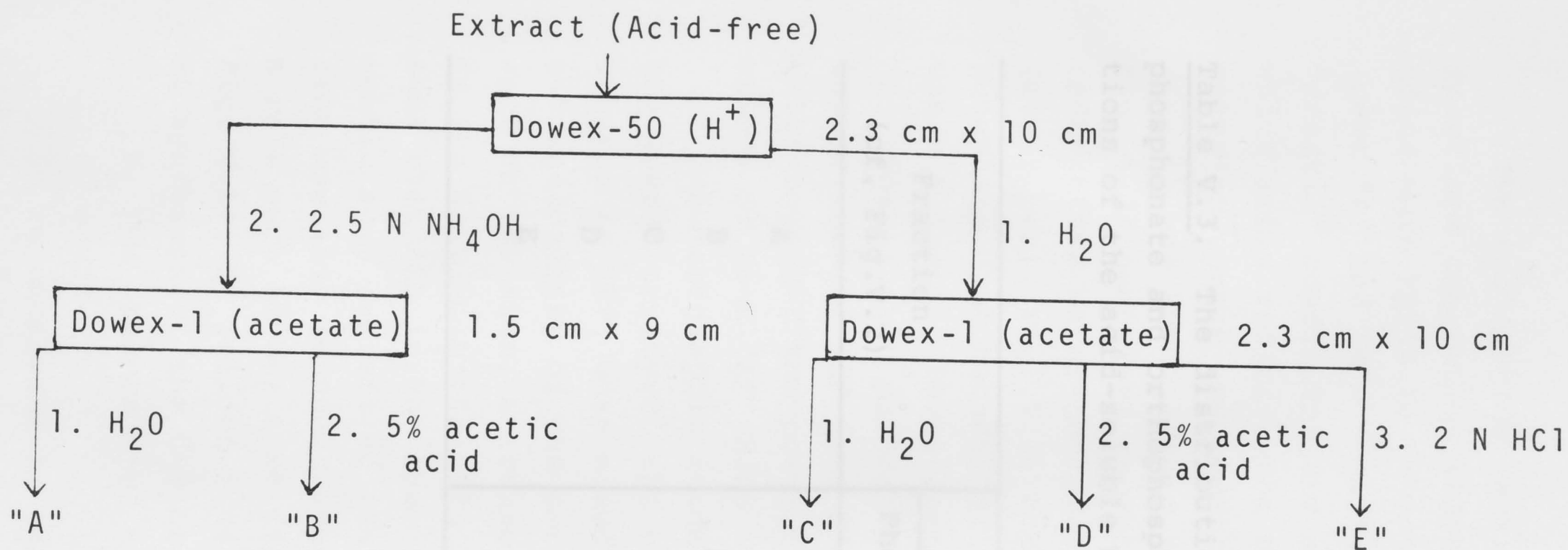


Fig. V.2. Schematic representation of the fractionation of the acid-soluble fraction from the slugs. Arrows indicate effluents or eluates which emerged in the order shown by numerals. In all cases, effluents were rendered solvent-free and the material was re-dissolved in water before applying to another column.

Two radioactive peaks emerged (Fig. V.3). The fractions comprising these peaks were pooled and the solutions were taken to dryness. Aliquots of the samples (peak "A" and "B", Fig. V.3) were hydrolysed under "drastic" conditions and examined for hydrolysis products which would indicate the original presence of radioactive

Table V.3. The distribution of the radioactivities of phosphonate and orthophosphate in hydrolysates of fractions of the acid-soluble material from the slug radioactive ester-phosphate being present. Peak "B" (minor peak, Fig. V.3) contained both phosphonate and

Fraction (cf. Fig.V.2)	Total $^{32}\text{P}$ radioactivity	
	Phosphonate	Ester-phosphate
A	-	1,000
B	200,000	85,000
C	2,500	1,500
D	1,250	3,750
E	-	$>10^7$

show that AEP in the free state is present in the trichloroacetic acid extract of the slug tissues. Since this fraction was also found to incorporate  $^{32}\text{P}$  orthophosphate at a rate much higher than lipid- or residue-bound AEP (cf. Fig. V.1), it follows that in the slug the site of primary biosynthesis of the C-P bond is a small molecule.

#### D. The precursor of the carbon skeleton of AEP in slugs

In an endeavour to gain further insight into the mechanism of C-P bond formation in slugs, the origin of

Two radioactive peaks emerged (Fig. V.3). The fractions comprising these peaks were pooled and the solutions were taken to dryness. Aliquots of the samples (peak "A" and "B", Fig. V.3) were hydrolyzed under "drastic" conditions and examined for hydrolysis products which would indicate the original presence of radioactive ester-phosphate and phosphonate.

It was found (Table V.4) that peak "A" (major peak, Fig. V.3) contained radioactive phosphonate only, no radioactive ester-phosphate being present. Peak "B" (minor peak, Fig. V.3) contained both phosphonate and phosphate, but because of the small amounts present, it was not further investigated.

The elution pattern of peak "A" (Fig. V.3) was similar to that of AEP (cf. Fig. III.5, Chapter III), and this suggested the possibility that the radioactive substance of peak "A" (Fig. V.3) was actually free AEP.

Chromatographic evidence (Fig. V.4) showed that, in the system employed, the compound from peak "A" (Fig. V.3) behaved as AEP, when mixed with authentic AEP. In which autoradiography of the chromatogram produced only one dark spot on the film, corresponding both in shape and position to the ninhydrin-staining area. These results clearly show that AEP in the free state is present in the trichloroacetic acid extract of the slug tissues. Since this fraction was also found to incorporate [ $^{32}\text{P}$ ]orthophosphate at a rate much higher than lipid- or residue-bound AEP (cf. Fig. V.1), it follows that in the slug the site of primary biosynthesis of the C-P bond is a small molecule.

#### D. The precursor of the carbon skeleton of AEP in slugs

In an endeavour to gain further insight into the mechanism of C-P bond formation in slugs, the origin of



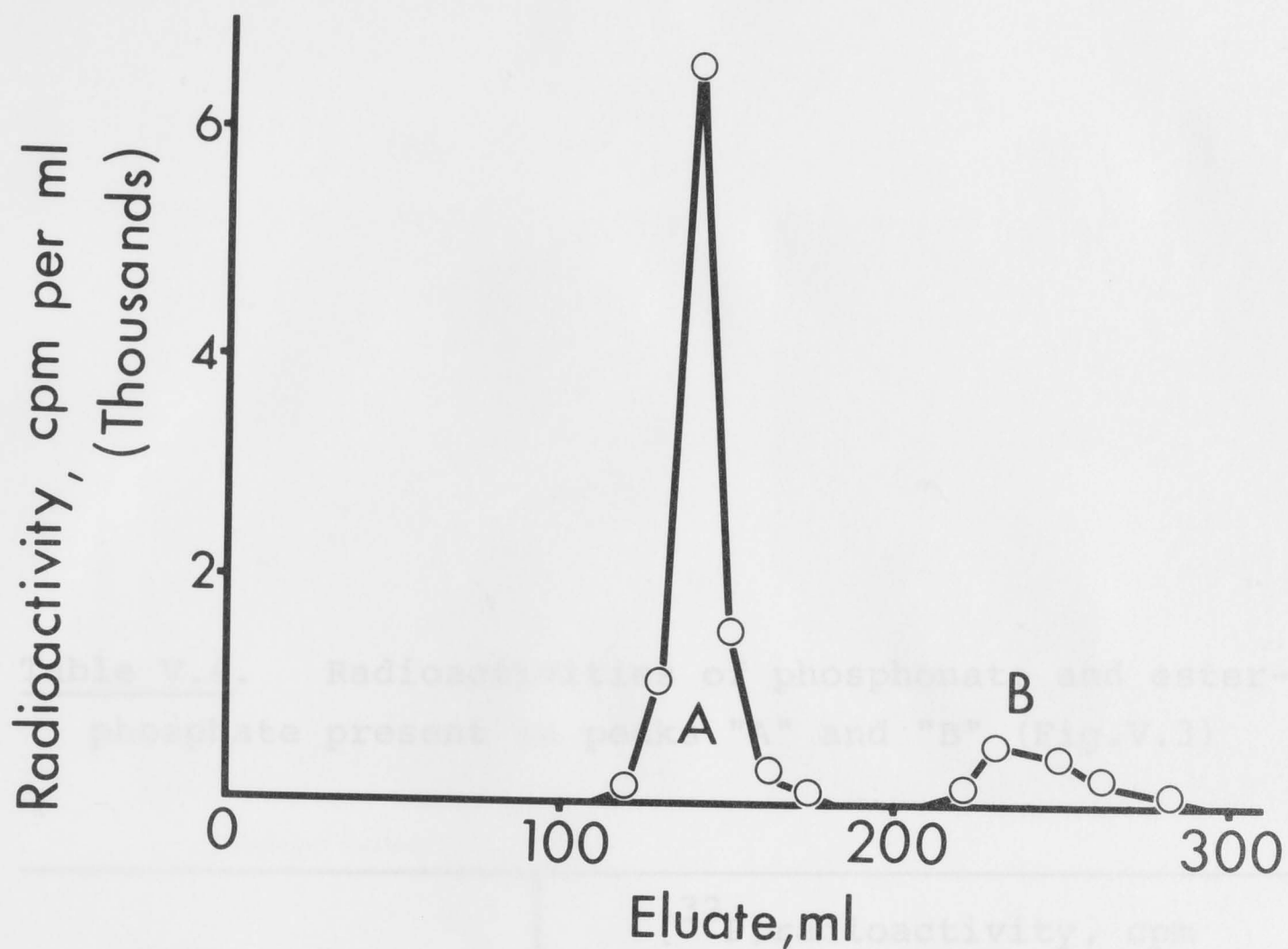


Fig. V.3. Emergence of radioactivity from Dowex-1 x 2 (formate) on chromatography of fraction "B" (Fig. V.2). An aliquot from fraction "B" was applied to a column (1.5 cm x 20 cm) of Dowex-1 x 2 (formate form, 200-400 mesh), and followed through with 50 ml of water. The column was then eluted with an increasing concentration gradient of formic acid, using 0.04 M formic acid in the main chamber and 300 ml of water in the mixing chamber. The effluent was collected in 10 ml fractions which were assayed for radioactivity.

Table V.4. Radioactivities of phosphonate and ester-phosphate present in peaks "A" and "B" (Fig.V.3)

Peak	[ <sup>32</sup> P]radioactivity, cpm	
	Phosphonate	Ester-phosphate
A	116,000	—
B	14,000	2,600

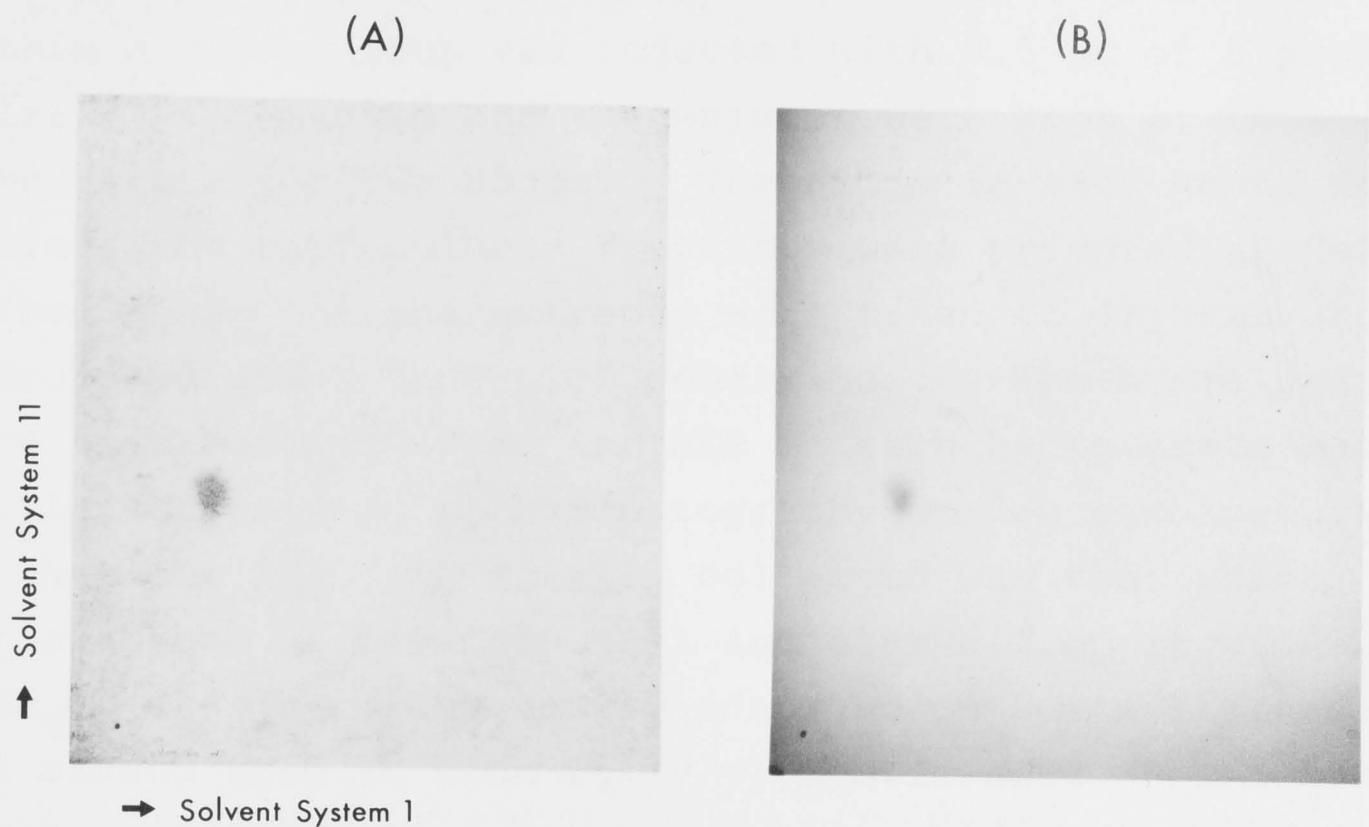


Fig. V.4. Two-dimensional paper chromatography of the material from peak "A" (Fig. V.3). Solvent systems as indicated. The sample from peak "A" was mixed with 1  $\mu$ mole of authentic AEP before it was applied to the paper. The compound was detected by; (A) ninhydrin; (B) autoradiography.

the carbon skeleton of AEP was investigated. Various [ $^{14}\text{C}$ ]compounds were tested for this purpose.

Fifteen slugs, of an average weight 0.8 g, were divided into five groups of three slugs each. Each slug within a given group was injected with 0.5  $\mu\text{C}$  of a particular [ $^{14}\text{C}$ ]compound and the animals were kept at room temperature for two hours. The slugs in each group were pooled, the acid-soluble fractions were prepared as described above and the extracts were taken to dryness and hydrolyzed under "drastic" conditions. The hydrolysates were rendered acid-free and AEP in each hydrolysate was partially purified by chromatography on ion exchange resin so that the fraction finally collected was that which (a) adsorbed on Dowex-50 ( $\text{H}^+$ ) and eluted from it with 2.5 N  $\text{NH}_4\text{OH}$  and (b) subsequently adsorbed on Dowex-1 (acetate) and eluted from it with 5% (v/v) acetic acid. The final eluate was taken to dryness and the residue dissolved in a small amount of water. Aliquots were subjected to paper electrophoresis (cf. "Methods", Chapter II) together with 5  $\mu\text{moles}$  of authentic AEP as a carrier. The dried chromatogram was dipped in 0.02% (w/v) ninhydrin in acetone and heated at  $80^\circ$  in an oven for 10 minutes. The AEP areas on the chromatogram were cut out and each paper strip was placed into a vial containing 5 ml of the "toluene" scintillator. The radioactivity was measured in the usual way (cf. "Methods", Chapter IV).

It was found (Table V.5) that neither of the two amino acids tested nor ethanolamine were efficient precursors of the carbon skeleton of AEP in slugs. The comparatively high degree of incorporation of [ $6\text{-}^{14}\text{C}$ ]glucose into AEP in contrast with that of [ $2\text{-}^{14}\text{C}$ ]acetate indicated that the carbon precursor of AEP may be an intermediate in glycolysis. This result was similar to that obtained with Tetrahymena (Chapter IV).



Table V.5. Incorporation of radioactivity from some [ $^{14}\text{C}$ ]-labelled compounds into AEP of the acid-soluble fraction of slugs

Experimental blank values used to correct the data were derived from counting the areas on the chromatogram directly adjacent to the AEP band.

$^{14}\text{C}$ -Carbon source injected			$^{14}\text{C}$ AEP isolated
Compound	Specific Radioactivity $\mu\text{C}$ per $\mu\text{mole}$	Total amount ( moles) injected into 3 slugs in each group	Total counts above blank
[2- $^{14}\text{C}$ ]Ethanolamine	3.57	0.42	470
[3- $^{14}\text{C}$ ]Serine	3.1	0.48	1,000
[2- $^{14}\text{C}$ ]Acetate	3.0	0.50	820
[6- $^{14}\text{C}$ ]Glucose	3.0	0.50	2,690
[U- $^{14}\text{C}$ ]Alanine	3.0	0.50	410

#### 4. Discussion

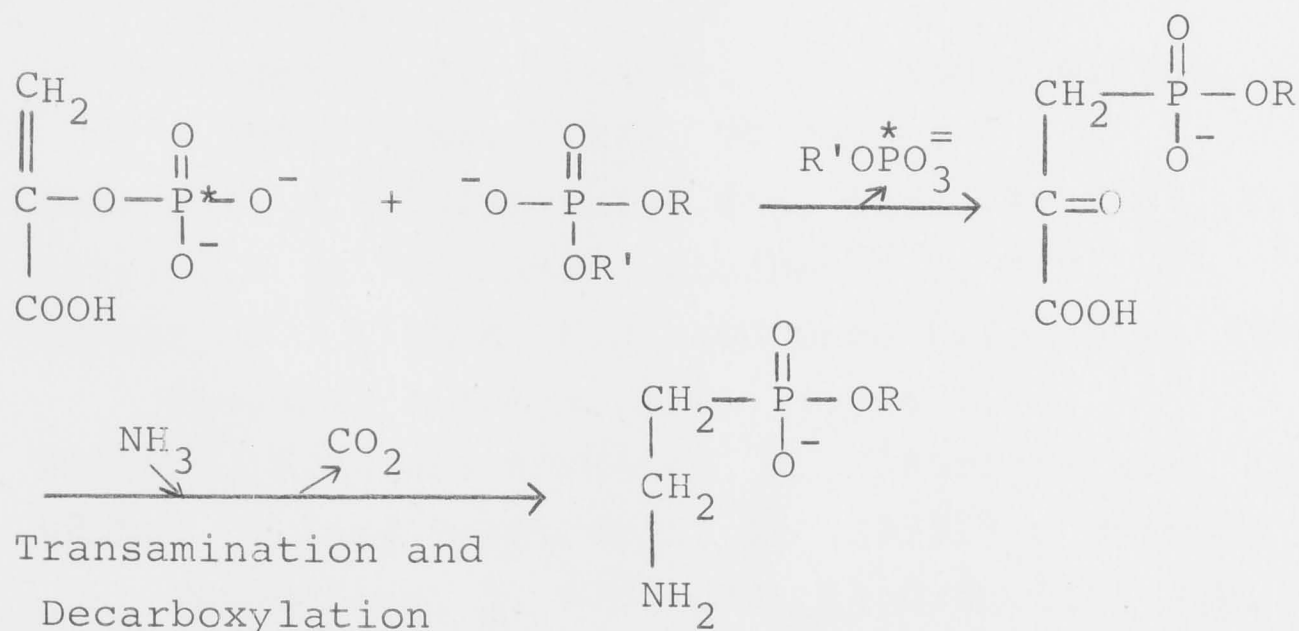
The existence of non-hydrolysable phosphorus compounds in slugs (Horiguchi, 1966) has now been confirmed and the compounds have been identified as AEP and its derivatives. The bulk of the AEP in slugs (60%) is found in the residual fraction. The lipid-bound and free AEP account for about 25% and 15% of the total, respectively. Quin (1965) reported that most of the phosphonate compounds of the bivalve molluscs Mytilus edulis and Venus mercenaria (quahog) could be extracted by aqueous ethanol. In each case AEP was isolated from the hydrolysates of these extracts. The AEP content of the residual fraction of the animals was reported to be negligible. This difference in distribution of phosphonate in the lipid and residual fractions of marine and terrestrial molluscs is of interest but a wider range of species must be examined before this can be accepted as part of a general pattern.

In slugs, the high specific radioactivity of the free AEP compared with the low specific radioactivity of AEP bound to lipid or other tissue elements indicates that the primary biosynthesis of the C-P bond in this species takes place in a small molecule with a high turnover rate. It is likely that the AEP of the lipid as well as that of the residual fraction originates from free AEP. The incorporation of free AEP into lipid may well occur through the familiar pathway (Kennedy and Weiss, 1956) in which cytidine nucleotide is involved (cf. Chapter III). The possible existence of these pathways in slugs was not further investigated. The incorporation of AEP into the macromolecules of the residual fraction may involve a complex biosynthetic system and nothing is at present known about this process.

Investigation of the possible precursor of the carbon skeleton of AEP in the slug yielded results similar to those obtained with Tetrahymena, and in both systems an intermediate in glycolysis is implicated as the precursor. The mechanism of C-P bond formation in these two organisms might be similar, except that the precursors of the phosphorus moiety are different. Results from Chapter II suggested that in Tetrahymena, the precursor of the phosphorus moiety of AEP resides in a glyceride molecule while in slugs, it is probably a small molecule (orthophosphate or a nucleotide).

One of the methods of chemical synthesis of phosphonates (cf. Chapter I) involves the condensation reaction of ethylene compounds (e.g. fumarate) with a phosphite ester or phosphorus pentachloride. In analogy, it may be suggested that phosphoenolpyruvate is the precursor of the carbon skeleton of AEP both in Tetrahymena (cf. Chapter IV) and in slugs.

The suggested scheme of C-P bond formation in Tetrahymena has been shown in Chapter IV (p.79) and that scheme could also be applied to the slugs, namely:



where R is diglyceride in Tetrahymena but it is simply a

proton in the slug. R' may be a nucleotide (cf. Lehmann and Benson, 1964).

The confirmation of the scheme relies on further experimental evidence.

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